Volume 23 September 1959 No. 5

BULLETIN OF THE AGRICULTURAL CHEMICAL SOCIETY OF JAPAN

ANALYTICAL CHEMISTRY ORGANIC CHEMISTRY BIOLOGICAL CHEMISTRY
FOOD & NUTRITION MICROBIOLOGY & FERMENTATION INDUSTRY
SOIL & FERTILIZER

CONTENTS

Analytical Chemistry

K. MORI and M. NAKAMURA: Quantitative Analysis of Sugars in Plant Extracts by Ion-exchange Chromatography, with Special Reference to the Examination of Conditions for preparing the Sample Sugar Solutions. 389

Organic Chemistry

H. UEDA, Y. SHIBAHARA and S. SHIMIZU: Studies on the Reduction of Terpenes with Sodium in Aqueous-ammonia. Part I. Synthesis of (−)-Menthol from (−)-Menthone. 376

H. UEDA and S. SHIMIZU: Studies on the Reduction of Terpenes with Sodium in Aqueous-ammonia. Part II. Synthesis of (+)-Isomenthol from Inverted Menthone. 380

N. TAKAHASHI, Y. SETA, H. KITAMURA and Y. SUMIKI: Biochemical Studies on "Bakanae" Fungus. Part 48. A New Gibberellin, Gibberellin A_4. 405

H. KITAMURA, Y. SETA, N. TAKAHASHI, A. KAWARADA and Y. SUMIKI: Biochemical Studies on "Bakanae" Fungus. Part 49. Chemical Structure of Gibberellins. Part XIV. 408

Y. SETA, N. TAKAHASHI, A. KAWARADA, H. KITAMURA and Y. SUMIKI: Biochemical Studies on "Bakanae" Fungus. Part 50. Chemical Structure of Gibberellins. Part XV. 412

S. MARUMO: Islanditoxin, a Toxic Metabolite produced by *Penicillium islandicum* Sopp. Part III. Structure of Islanditoxin. 428

Biological Chemistry

F. YOSHIDA, M. NAGASAWA and E. ICHISHIMA: Studies on the Proteolytic Enzymes of Black Aspergilli. Part V. The Specificity of Crystalline *Asp. Saitoi* Proteinase by Application of the Sanger DNP-Method. 363

I. CHIBATA and T. TOSA: Studies on Amino Acids. XII. Studies on the Enzymatic Resolution (X): Acylase Activity in Plants. 370

(*continued on cover* 3)

Published by the Agricultural Chemical Society of Japan
c/o Faculty of Agriculture, University of Tokyo

THE AGRICULTURAL CHEMICAL SOCIETY OF JAPAN
(NIPPON NÔGEI-KAGAKU KAI)

President
Takajirô MORI

Vice Presidents
Genshirô KAWAMURA   Jirô NIKUNI

EDITORIAL BOARD

Chairman: Kin-ichirô SAKAGUCHI

Hisashi ARIYAMA
Toshinobu ASAI
Fujio EGAMI
Yoshiyuki INOUE
Hisayoshi IWATA
Hideo KATAGIRI
Nobuhiko MIGITA
Ziro NIKUNI
Yatarô OBATA
Azuma OKUDA
Yasuyoshi ÔSHIMA
Yoshito SAKURAI
Rinjirô SASAKI
Toriji SASAKI
Yusuke SUMIKI
Isamu TACHI
Sankichi TAKEI
Teiichi TAMURA
Izue YAMASAKI

Advisory Editors:
Kakuji GOTÔ
Kinsuke KONDÔ
Yuzuru OKUDA
Teijirô YABUTA

Managing Editors:
Kôichi YAMADA   Masanao MATSUI

The Bulletin of the Agricultural Chemical Society of Japan is published bimonthly to provide original papers on agricultural chemistry.

Manuscripts submitted for publication and all inquiries regarding them should be sent to the Editor of the Bulletin, The Agricultural Chemical Society of Japan, c/o Departments of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

Annual subscription price is $6.00 for all oversea subscribers including postage fee. Please address order to the agencies.

The Agricultural Chemical Society of Japan also published The Journal of the Agricultural Chemical Society of Japan (Nippon Nôgei-Kagaku Kaishi), printed in Japanese.

AGENCIES

Charles E. Tuttle Co.
Rutlant, Vt.
U.S.A.

Maruzen Co., Ltd.
P. O. Box 605, Tokyo Central
Tokyo, Japan.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 351～358, 1959]

Studies on Osmophilic Yeasts

Part V. Factors Affecting Growth of Soy Yeasts and Others in the Environment of a High Concentration of Sodium Chloride. (II)

By Hiroshi Ōnishi

Noda Institute for Scientific Research, Noda City, Chiba-ken

Received November 18, 1958

The influences of temperature on growth and death of osmophilic yeasts in environments of both ordinary and high concentrations of sodium chloride were investigated. It was found that many osmophilic yeasts are able to grow in saline medium at a high temperature such as 40°, whereas in NaCl-free medium no growth occurred at the same temperature. Effects of various factors on this phenomena were examined. This halophilic property of yeast cells at a high temperature seemed to be due to the active metabolism of the cells in such an environment but not to the increase of heat-stability of cell proteins or enzymes that might be caused by the dehydration by effect of sodium chloride.

Specific aspects of metabolism of the osmophilic yeasts in two comparative environments of ordinary and high saline have been studied by the author[1,2]. In the previous report[1], the nutritional requirements of the osmophilic yeasts in environments of both an ordinary (NaCl-free) and a high concentration of sodium chloride (NaCl 18%) were examined and the influences of pH of the medium upon the growth in both environmental conditions were also described.

In this paper, the influences of temperature on growth and death of the osmophilic yeasts in these comparative environments were observed and it was found that maximum growth temperature of the osmophilic yeasts was increased from 32°～35° to 40° by cultivation in the saline medium. The explanation of this effect is not completely clarified, but the author may be allowed to point out one possibility here.

EXPERIMENTAL AND RESULTS

[I] Effects of Temperature on Growth of Osmophilic Yeasts in the NaCl-free and 18% NaCl Medium.

From observations on the growth of *Saccharomyces rouxii*, which played an important role in soy-brewing, in NaCl-free and 18% NaCl koji extract at various temperatures, it was found that this yeast is able to grow at 40° in the 18% NaCl medium though no growth occurred at the same temperature in the ordinary plain medium (see Fig. 2). Then, it was examined whether or not many other osmophilic yeasts show the same halophilic property at 40°. From the results given in Table I, this halophilic property at a high temperature seemed to be common in most of the osmophilic yeasts tested.

[II] Factors which support the Growth of *Saccharomyces rouxii* at a High Temperature.

(1) Effects of sodium chloride or glucose concentrations of the medium.

The minimum effective concentration of sodium chloride to support the growth of *Sacch. rouxii* at 40°, was first examined. As shown in Table II, *Sacch. rouxii* cells were able to multiply at 40° in the presence of sodium chloride at concentrations exceeding 3～4%, and similarly in the presence of glucose with a further addition of more than 15～20% to the basal medium.

(2) Effect of the conditions of preculture.

When cells of *Sacch. rouxii* grown in the 10% NaCl koji extract at 30° or 40°, were transferred to the NaCl-free koji extract, no growth occurred at 40°. Therefore, adaptation of this yeast to a higher temperature by cultivation in the high saline medium was unsuccessful.

---

[1] H. Ōnishi, This Bulletin, **21**, 143 (1957).

[2] H. Ōnishi, This Bulletin, **23**, 332 (1959).

TABLE I. EFFECT OF A HIGH CONCENTRATION OF SODIUM CHLORIDE ON THE GROWTH OF VARIOUS OSMOPHILIC YEASTS AT THE HIGH TEMPERATURES

(15 days cultivation in the NaCl 0% and 18% koji extract)

Strains		30°		35°		40°	
		NaCl 0%	18%	0%	18%	0%	18%
Sacch. rouxii	m1	+++	++			−	++
〃	m2	+++	++	+++	++	−	−
〃	m4	+++	++			−	+
〃	m5	+++	+++			−	+
〃	m6	+++	++			−	±
〃	m8	+++	+++			−	+
〃	m9	+++	+++			−	++
〃	m11	+++	+++			−	+++
〃	s5	+++	+++			−	+++
〃	s7	+++	++			−	+
〃	s8	+++	+++	−	+++	−	−
〃	N5	+++	+++			−	++
〃	N8	+++	+++			−	++
〃	N28	+++	++			−	++
〃	A34	+++	++			−	++
S. rouxii var. *halomembranis*	A31	+++	++			−	++
〃	sal 4	+++	++			−	+
〃	H5	+++	++			−	+
〃	j3	+++	++			−	−
〃	sal 6	+++	++			−	±
S. acidifaciens	s9	+++	++			−	++
S. acidifaciens var. *halomembranis*	H3	+++	++			−	+
Torulopsis nodaensis	N21	+++	+++	+	+++	−	−
Torulopsis halophilus	N24	+++	+++	−	+++	−	−
Torulopsis versatilis	F5	+++	+++	−	++	−	−
Pichia farinosa	A6	+++	+++			+++	+++
Debaryomyces hansenii	H70	+++	+++			+++	+++

TABLE II. EFFECTS OF THE CONCENTRATIONS OF SODIUM CHLORIDE OR GLUCOSE ON THE GROWTH OF *Sacch. rouxii* AT 40°

Basal medium: koji extract (direct reducing sugar 10% as glucose) 40°, 10 days

Concentration of NaCl or glucose added to the basal medium	*S. rouxii* m 1	*S. rouxii* m 8	*S. rouxii* m 11
0	−	−	−
NaCl 3%	+	−	−
NaCl 6%	++	++	++
NaCl 9%	++	++	++
glucose 5%	−	−	−
glucose 10%	−	−	−
glucose 15%	±	−	+
glucose 20%	++	++	++
glucose 30%	++	++	++

(3) Effect of vigorous aeration.

Since Tanner and Wallace[3], and also Imšenecki and Solnzeva[4] have concluded that limitation of growth in cultures of thermophilic bacteria is very probable due to exhaustion of the oxygen supply, the effect of aeration on the growth of this yeast at 40° was investigated. However, there was practically no difference in growth at 40° between the stationary culture and the shake culture; cells of *Sacch. rouxii* failed to grow in both cultures at 40° in the NaCl-free medium.

(4) Effect of addition of various substances.

As substances that prevent denaturation of proteins by heating, sodium caprylate[5] sodium dodecylsulfate[6],

---

[3] F. W. Tanner and G. I. Wallace, *J. Bact.*, **10**, 421 (1925).

[4] A. Imšenecki and L. Solnzeva, *J. Bact.*, **49**, 539 (1945).

[5] P. D. Boyer et al., *J. Biol. Chem.*, **162**, 181 (1946).

[6] E. L. Duggan and J. M. Luck, *J. Biol. Chem.*, **172**, 205 (1948).

TABLE III. EFFECT OF ADDITION OF VARIOUS SUBSTANCES ON THE GROWTH OF *Sacch. rouxii* AT 40°

The Basal Medium: Bllg. 10° koji extract

Amounts of Substances added to the Basal Medium	*S. rouxii* m 1	*S. rouxii* m 8	*S. rouxii* m 11
NaBr 1 Mol	+++	++	+++
$NaNO_3$ 1 Mol	++	++	++
NaCl 1 Mol	++	++	++
KBr 1 Mol	++	++	++
KNO_3 1 Mol	++	++	++
KCl 1 Mol	+++	++	++
$MgCl_2 \cdot 6H_2O$ 0.5 Mol	++	++	++
Saccharose 10%	−(+++)	−(+++)	−(+++)
〃 20%	−(+++)	−(+++)	−(+++)
〃 30%	++	++	++
〃 40%	++	++	++
Galactose 30%	++	++	++
Glycerin 1 Mol	+	±	+
〃 2 Mol	++	++	++
〃 3 Mol	++	++	++
Soluble starch 2%	−(+++)	−(+++)	−(+++)
〃 3%	−(+++)	−(+++)	−(+++)
Na-alginate 0.5%	−(+++)	−(+++)	−(+++)
〃 1.0%	−(+++)	−(+++)	−(+++)
Agar 0.1%	−(+++)	−(+++)	−(+++)
〃 0.05%	−(+++)	−(+++)	−(+++)
Peptone (Total N: 1.5%)	−(+++)	−(+++)	−(+++)
soy-bean oil 1%	−(+++)	−(+++)	−(+++)
Na-caprylate 10^{-3}Mol	−(+)	−(+)	−(+)
〃 5×10^{-4}Mol	−(++)	−(+)	−(++)
Na-dodecylsulfate 3×10^{-3}Mol	−(−)	−(−)	−(−)
〃 5×10^{-4}Mol	−(+++)	−(+++)	−(+++)
〃 10^{-4}Mol	−(+++)	−(+++)	−(+++)
Congo red 10^{-3}Mol	−(+++)	−(+++)	−(+++)
〃 10^{-4}Mol	−(+++)	−(+++)	−(+++)
〃 10^{-5}Mol	−(+++)	−(+++)	−(+++)
Control (no addition)	−(+++)	−(+++)	−(+++)

( ) indicates the growth at 30°.

congo red[7)] and concentrated solutions of sugars[8),9)] have been reported. In addition to these substances, effects of various sodium and potassium salts, soluble starch, sugar, glycerol, sodium alginate, agar, soy-bean oil and a concentrated solution of polypeptone on growth of these yeasts at 40° were investigated.

As shown in Table III, one molar concentration of NaBr, $NaNO_3$, KNO_3 and KCl showed a similar effect as one mole of NaCl. Addition of saccharose or galactose with concentrations higher than 30% was also effective in supporting growth. However, the addition of agar (0.1%), sodium alginate (1.0%), soy-bean oil (1%), or polypeptone (1.5% as total N), was found to be ineffective in supporting growth of these yeasts at 40°.

Sodium caprylate, sodium dodecylsulfate and congo red which were generally accepted as the inhibitor of heat denaturation of proteins, were also found ineffective.

---

7) F. Haurowitz, Chemistry and Biology of Protein, p. 132 (1950).

8) A. C. Fay, *J. Agr. Research.*, **48**, 453 (1934).

9) T. Brosteaux and B. Erickson-Quensel, *Arch. Phys. Biol.*, **12**, 209 (1935).

TABLE IV. EFFECT OF NITROGEN SOURCES AND VITAMINS ON THE GROWTH OF *Sacch. rouxii* AT 40°

Media	% of NaCl or glucose added to the basal medium		*S. rouxii* m 8		*S. rouxii* m 11	
			30°	40°	30°	40°
The $(NH_4)_2SO_4$ medium		0	+++	−	+++	−
	NaCl	1M	+++	++	+++	++
	〃	1.5M	+++	++	+++	++
	〃	2M	+++	++	+++	++
	Glucose	10%	+++	−	+++	−
	〃	15%	+++	−	+++	−
	〃	20%	+++	+	+++	++
The casamino acid medium		0	+++	−	+++	−
	NaCl	1M	+++	++	+++	++
	〃	1.5M	+++	++	+++	++
	〃	2M	+++	++	+++	++
	Glucose	10%	+++	−	+++	−
	〃	15%	+++	++	+++	++
	〃	20%	+++	++	+++	++
Thiaminie omitted medium		0	+++	−	+++	−
	NaCl	1M	+++	++	+++	++
	〃	1.5M	+++	++	+++	++
	〃	2M	+++	++	+++	++
	Glucose	10%	+++	−	+++	−
	〃	15%	+++	−	+++	−
	〃	20%	+++	++	+++	++
Inositol omitted medium		0	+++	−	+++	−
	NaCl	1M	+++	+	+++	++
	〃	1.5M	+++	++	+++	++
	〃	2M	+++	++	+++	++
	Glucose	10%	+++	−	+++	−
	〃	15%	+++	−	+++	−
	〃	20%	+++	++	+++	++
riboflavin, pyridoxine, niasin, and *p*-amino-benzoic acid omitted medium		0	+++	−	+++	−
	NaCl	1M	+++	++	+++	++
	〃	1.5M	+++	++	+++	++
	〃	2M	+++	++	+++	++
	Glucose	10%	+++	−	+++	−
	〃	15%	+++	±	+++	++
	〃	20%	+++	++	+++	++

(5) Effects of nitrogen sources and vitamins.

In the previous paper[1], it has been found that *Sacch. rouxii* could similarly utilize both ammonium salts and amino acids in these two contrasted environments of NaCl 0% and 18% provided the pH of medium were suitable.

Influence of the nutritional conditions on growth of these yeasts at 40° was examined.

The composition of the basal medium was as follows: glucose 5%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.01%, Vitamins (biotin 2 μg, Ca-pantothenate 400 μg, inositol 2000 μg, niacin 400 μg, *p*-aminobenzoic acid 200 μg, pyridoxine 400 μg, thiamin 400 μg, riboflavin 200 μg were added per 1 l of the medium). To 1 l of this medium, 25 cc of a buffer solution (100 g of potassium citrate and 25 g of citric acid per 1 l) was added. As nitrogen source, ammonium sulfate or vitamin free casamino acid (Difco) was added

TABLE V. INFLUENCE OF THE MINERAL REQUIREMENTS ON THE GROWTH OF *Sacch. rouxii* AT 40° (14 days)

Media	30°	40°
Basal medium	+++	−
10% NaCl basal medium	+++	++
Basal medium added with (1)* of trace elements	+++	−
Basal medium added with (2)* of trace elements	+++	−
Basal medium added with $MnSO_4 \cdot 4H_2O$ 0.04%	+++	−
Basal medium added with $FeCl_3 \cdot 6H_2O$ 0.02%	+++	−
Basal medium added with $ZnSO_4 \cdot 7H_2O$ 0.04%	+++	−
Basal medium added with $Co(NO_3)_2 \cdot 6H_2O$ 0.01%	+++	−

The basal medium : "Difco" yeast nitrogen base added with 5% glucose

* Amounts of elements per 100cc of the medium

	(1)	(2)
H_3BO_3	500 μg	5 mg
$CuSO_4 \cdot 5H_2O$	40 μg	400 μg
KI	100 μg	1 mg
$FeCl_3 \cdot 6H_2O$	200 μg	2 mg
$MnSO_4 \cdot 4H_2O$	400 μg	4 mg
$Na_2MoO_4 \cdot 2H_2O$	200 μg	2 mg
$ZnSO_4 \cdot 7H_2O$	400 μg	4 mg

TABLE VI. EFFECT OF SODIUM CHLORIDE ON THE THERMAL DEATH TIME OF *Sacch. rouxii*

Strains	NaCl concentration of the media	Heating condition							
		50°				55°			
		10 min	20 min	60 min	120 min	1 min	2 min	15 min	20 min
S. rouxii m 8	0%	++	−−	−−	−−	+−	−−	−−	−−
	18%	++	++	++	−−	++	++	++	−−
S. rouxii m 11	0%	++	−−	−−	−−	−−	−−	−−	−−
	18%	++	++	++	++	++	++	++	++

to the basal medium in an amount of 0.03% as total nitrogen.

Effects of vitamins were tested by employing a medium in which each vitamin was omitted one by one. In this case, biotin and Ca-pantothenate were always added because they are essential for growth of the yeasts tested.

From the results shown in Table IV, it was revealed that when either ammonium sulfate or casamino acids were employed as the nitrogen source, the yeasts were able to grow at 40°, only in the media added with NaCl (1 Mol or more) or glucose (more than 15%) but not in the plain medium, and that there were no distinct differences in the minimum effective concentration of sodium chloride to support growth of these yeasts at 40° even when each one or all of the non-essential vitamins were omitted from the basal medium.

(6) Mineral requirement.

Effects of larger amounts of the trace elements, such as B, Cu, Fe, I, Mn, Mo, Zn and Co, 10∼100 times as much as those in the ordinary medium were tested. The media employed and the results obtained are shown in Table V. The addition of these elements were all ineffective.

[III] Effect of Sodium chloride on the Thermal Death Time of *Saccharomyces rouxii*.

From the above results, it was expected that thermal death-time is elongated in the presence of sodium chloride. From the following experiment, it was confirmed that the above expectation is correct. One drop of yeast suspension was inoculated into the plain koji-extract and the 18% NaCl koji-extract in the test tubes. Attention was payed so as not to let the cell suspension come into contact with the wall of the test tubes. After keeping the tubes for a given-time period at each temperature, they were cooled at once below 30° and incubated at 30° for 14 days. The test was carried out in duplicate (Table VI). From the results demonstrated in Table VI, it would be apparent that the thermal death-time of cells is elongated about tenfolds in the saline medium as compared with that in the plain medium.

[IV] The Growth Curve and the Death Curve of *Saccharomyces rouxii* at 30° and 40° in both the Plain and Saline Media.

Viable count of *Sacch. rouxii* cells which were incubated in the plain and 10% NaCl koji extract at 30° and 40°, was carried out. The growth curve and the death curve in each of the two media are illustrated in Fig. 1. In this example, the following seven media were employed as plating ones.: ①: koji extract agar, ②: the synthetic medium (glucose 5%, KH_2PO_4 0.1%, $MgSO_4\cdot7H_2O$ 0.05%, $CaCl_2\cdot2H_2O$ 0.01%, NaCl 0.01%, Vitamin-free casamino acid 0.4%, Yeast-extract 0.1%, Agar 2%), ③ : ②+Na-acetate 0.2%, ④ : ②+K-citrate 0.2%, ⑤ : ②+Na-pyruvate 0.2%, ⑥ : ②+DL-α-alanine 0.4%, ⑦ : ②+extract of *Sacch. rouxii* cells which were cultivated in the 18% NaCl medium.

Similar growth and death curves were obtained in all of the seven plating media, showing no significant difference in the effects of these plating media on recovery from death.

[V] The Growth Curve and the Exponential Growth Rate Curve of *Saccharomyces rouxii* at Various Temperatures.

The growth curves of 2 strains of *Saccharomyces rouxii* at various temperatures in the 0% NaCl and 10% media are shown in Fig. 2. The medium employed was the same as that of [II] (5); ammonium sulfate was the nitrogen source and potassium citrate-citric acid mixture was the buffer in the medium. The extent of growth was measured turbidimetrically with a spectrophotometer. Exponential growth rates of these yeasts at various temperatures were calculated from growth curves shown in Fig. 2, and the relative values were illustrated in Fig. 3.

The exponential growth rate curve in the NaCl-free medium rapidly fell down at temperature of 32°～35° which was only 2°～3° higher than optimum growth temperature, and in the 10% NaCl medium the exponential growth rate curve did not fall down near to 40°～42.5°, though shapes of these curves in both media were similar. This seemed to explain that death of the yeast cells at higher temperatures such as 35°～40° may be caused by the inactivation of enzymes and the denaturation of proteins, and sodium chloride may apparently protect this inactivation.

[VI] Influences of the Energy Sources on Viability of *Saccharomyces rouxii* at Various Temperatures in the Plain and the Saline Medium.

Well-washed yeast cells of the fresh culture of *Sacch. rouxii* were suspended into the following four media and incubated at 10°, 30°, and 40°. Viability of the yeast cells was determined by plating on koji agar.

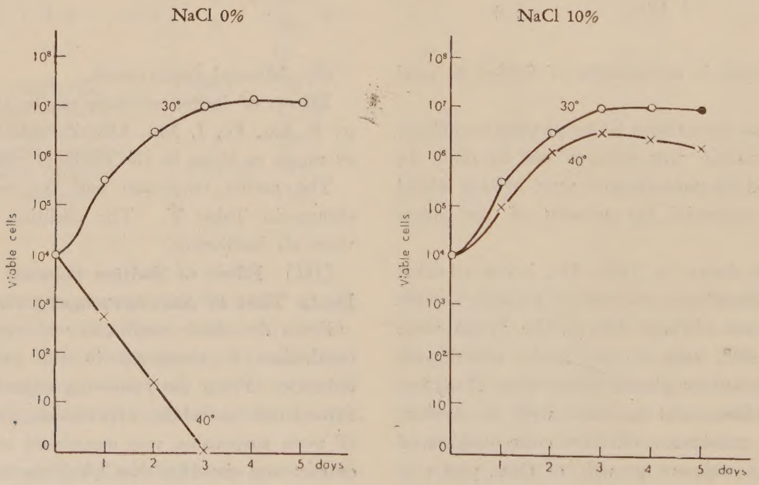


FIG. 1. The Growth Curve and the Death Curve of *Sacch. rouxii* at 30° and 40° in Both Media of the Plain and the Saline

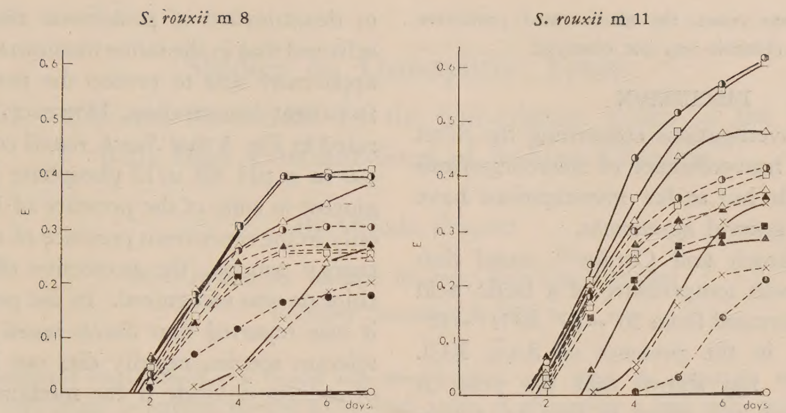


FIG. 2. The Growth Curve of *Sacch. rouxii* at Various Temperatures

○ 15° × 20° △ 25° □ 30° Solid line: NaCl 0%
◑ 32.5° ▲ 35° ■ 37.5° ● 40° Broken line: NaCl 10%

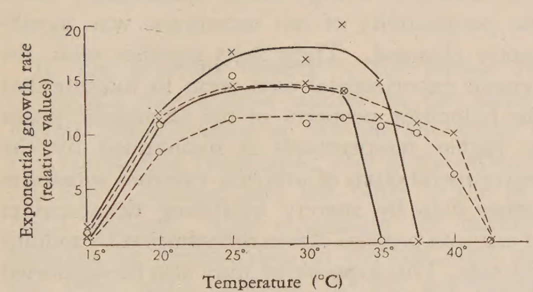


FIG. 3. The Exponential Growth Rate Curve of *Sacch. rouxii* at Various Temperatures

○: *Sacch. rouxii* m 8 Solid line: NaCl 0%
×: *Sacch. rouxii* m 11 Broken line: NaCl 10%

The suspending media employed were as follows: ①: pH 4.8 M/15 phosphate buffer, ②: NaCl 10% in pH 4.8 M/15 phosphate buffer, ③: glucose 5%, in pH 4.8 M/15 phosphate buffer, ④: NaCl 10% and glucose 5% in pH 4.8 M/15 phosphate buffer.

The results obtained are shown in Fig. 4, in which it was demonstrated that although the sole addition of 10% NaCl showed no protective effect on cell of this yeast, sodium chloride brought about a good result in maintaining viability of yeast cells in the presence of those substances such as glucose which are easily utilizable by yeasts as energy sources.

In the presence of lactose or xylose which could not

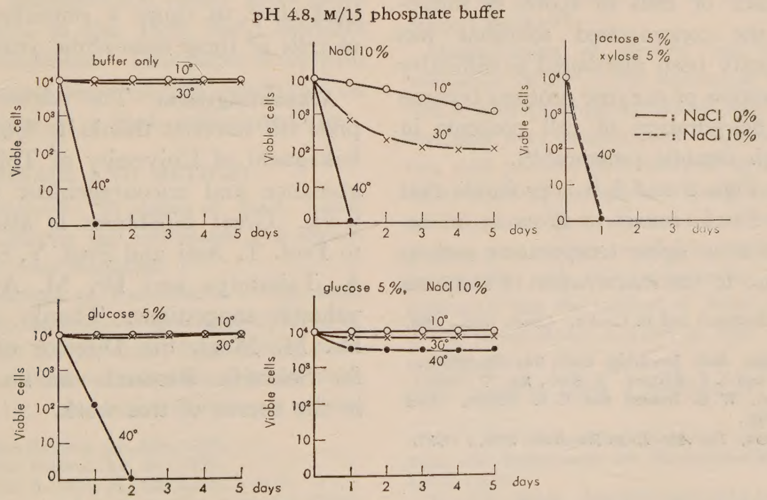


FIG. 4. Effect of the Energy Sources on Viability of *Sacch. rouxii* at Various Temperatures in the Plain and the Saline Medium

be utilized by these yeasts, the above-stated protective effect of sodium chloride was not observed.

DISCUSSION

Numerous investigations concerning the effect of salts on the heat-tolerance of microorganisms have been made but so far investigations have not reached a general agreement.

Richet, Bachrach and Cardot[10] stated that maximum growth temperature of a lactic acid bacterium is increased from 36°~37° to 41°~42° by cultivation in the presence of 3.4% KCl. van Halteren[11] has shown that the yeast is protected against the effect of heat by a concentrated solution of salts or sugars. On the other hand, Casman and Rettger[12] failed to find any protective effect of hypertonic solutions, such as 20~40% peptone, 10~15% NaCl, and 25~35% lactose on a number of organisms of the *Bac. subtilis* group. Anderson et al.[13] found that glucose and sucrose increase the heat resistance of *Bac. coagulans*, while NaCl and several organic acids have a decreasing effect.

It appears that the effect of salts is variable depending upon concentration, the kind of suspension medium and the test organism used.

The high resistance of dry proteins to heat denaturation is well known. Thus, the increased thermal resistance of cells or spores of microorganisms in the concentrated solutions has hitherto principally been attributed to difficulty of heat denaturation of enzyme proteins because of the actual dehydration of cell contents in solutions of high osmotic pressure[8,14].

As shown in Figs. 2 and 3, it is probable that the failure of *Sacch. rouxii* to grow in an ordinary medium at a higher temperature such as 35° to 40° is due to the inactivation of enzymes or denaturation of proteinous molecules in the cells and that in the saline medium sodium chloride apparenlty acts to protect the protein molecules from heat denaturation. However, it was demonstrated in Fig. 4 that *Sacch. rouxii* cells rapidly die out in a pH 4.8 M/15 phosphate buffer without glucose in spite of the presence of 10% NaCl, and only in the concurrent presence of easily utilizable energy sources, the protective effect of sodium chloride was recognized. In the previous paper[2], it was reported that *Sacch. rouxii*, a typical salt-tolerant species, rapidly dies out in the presence of sodium chloride if the medium is devoid of the easily utilizable energy sources, and that active metabolism is indispensable for maintaining viability in a high saline medium, in which the permeability of cell membrane was significantly changed. These facts together with the present experimental data seem to indicate that the halophilic property of the osmophilic yeasts at higher temperatures is maintained by the active metabolism of utilizing external substrates rather than by merely inhibiting denaturation of enzyme proteins due to dehydration by sodium chloride. This supposition may also be supported by the fact that this halophilic phenomena at higher temperatures occurs in a saline environment as low as 3~6% NaCl, which seems to be ineffective to cause a remarkable dehydration of cells of these osmophilic yeasts.

Acknowledgement The author wishes to express his sincerest thanks to Emeritus Prof. K. Sakaguchi of University of Tokyo for his kind guidance and encouragement throughout this work. Great indebtness is also acknowledged to Prof. T. Asai and Prof. Y. Sumiki and Prof. A. Takamiya and Dr. M. Amaha for their valuable suggestions. Thanks are also due to Dr. M. Mogi, the Director of Noda Institute for Scientific Research for his encouragement in the cource of this work.

---

[10] C. Richet, E. Bachrach and H. Cardot, *Compt. rend.*, **180**, 1997 (1925).

[11] van P. Halteren, *Bull. Soc. Chim. Biol.*, **32**, 458 (1950).

[12] E. P. Casman and L. F. Rettger, *J. Bact.*, **26**, 77 (1933).

[13] E. E. Anderson, W. B. Esselen and C. R. Fellers, *Food Research* **14**, 499 (1949).

[14] A. H. Robertson, *Ut. Agr. Expt. Sta. Bull.*, **274**, 1 (1927).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 359~363, 1959]

Studies on Osmophilic Yeasts

Part VI. Glycerol Production by the Salt-tolerant Yeasts in the Medium with High Concentrations of Sodium Chloride

By Hiroshi Ōnishi

Noda Institute for Scientific Research, Noda City, Chiba-ken

Received December 9, 1958

Saccharomyces rouxii which is a typical salt-tolerant yeast and plays an important role in soy-brewing, was found to ferment glucose giving high yields of glycerol in a high saline meduim under aerobic conditions, though only a small amount of glycerol was produced in the ordinary medium. An amount as much as 40~50% of glucose fermented was converted to glycerol under the above conditions.

In the previous reports[1,2], the author has conducted comparative studies on the physiological characteristics of salt-tolerant yeasts in two contrasting conditions; one, in the ordinary NaCl-free medium and the other in the 18% NaCl medium. After examining the material balances for fermentation in the NaCl-free and the 18% NaCl medium by the salt-tolerant yeasts of various genera and species[3,4,5] which were isolated from soy-mashes, the present author has found that *Saccharomyces rouxii* gives high yields of glycerol in the 18% NaCl medium under aerobic conditions. This paper deals with the effects of various fermentation conditions on glycerol production in a high saline medium.

MATERIALS AND METHOD

Yeast strains employed: 29 strains of *Sacch. rouxii*, isolated from soy-mashes and miso-pastes and showed high salt-tolerance, were employed.

Media and conditions of fermentation: The basal medium composition was follows: glucose 10%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.01%, vitamin-free casamino acid (Difco) 0.4%, Yeast extract (Difco) 0.1%. Various modifications of this medium were made by using different concentrations of salts and by adding various kinds of nitrogen sources. In the stationary and anaerobic cultures, 100 ml of these media were placed in 150 ml Erlenmyer flasks, sterilized, inoculated and incubated at 30°. In the aerobic culture, 80 ml of these media were placed in 500 ml shaking flasks, and the flasks were shaken on a reciprocal shaker operating at 120 r.p.m. with a stroke of 75 mm at 30°. After 10 days' incubations, the fermentation products were analysed.

Analytical method:

Glucose was determined by the Fehling-Lehmann-Schoorl method[6].

Determination of glycerol was conducted by the method of Neish[7]. The amouct of crude glycerol, in which other polyhydric alcohols such as mannitol and erythritol were contaminated, was determined by oxidation with periodic acid followed by colorimetric determination of formaldehyde formed with a chromotropic acid reagent. The values obtained were corrected for glucose presented by running a standard glucose sample in parallel with the fermentation solutions. It was confirmed that the method of Neish is fully applicable to the fermentation solution of 18% NaCl without any modification. Pure glycerol was separated on water-celite columns using a mixture of 25% benzene and

---

[1] H. Ōnishi, This Bulletin, **21**, 143 (1957).

[2] H. Ōnishi, This Bulletin, **23**, 351 (1959).

[3] H. Ōnishi, This Bulletin, **21**, 151 (1957).

[4] H. Ōnishi, Lecture read at the meeting of the Agr. Chem. Soc. of Japan (April 10, 1957).

[5] H. Ōnishi, This Bulletin, in press.

[6] A. W. van der Haar, Anleitung zum Nachweis, zur Trennung und Bestimmung der Monosaccharide und Aldehydsäuren, Berlin (1920).

[7] A. C. Neish, Analytical method for bacterial fermentations. 2nd. rev. Natl. Research Council Can., Prairie Regional Lab., Saskatoon, Sask. N.R.C. No. 2952 (1952).

TABLE I. GLYCEROL PRODUCTION BY *Sacch. rouxii* IN THE NaCl-FREE AND 18% NaCl MEDIA UNDER AEROBIC AND ANAEROBIC CONDITIONS

Cultural conditions	Strains	The NaCl-free medium			The 18% NaCl medium		
		% of glucose consumed	% of crude glycerol formed	yield of glycerol on glucose consumed	% of glucose consumed	% of crude glycerol formed	yield of glycerol on glucose consumed
Anaerobic*	N 28	6.12	0.457	7.4%	6.05	0.511	8.4%
	E 7	6.54	0.495	7.5	5.86	0.614	10.5
Stationary*	N 28	5.24	0.409	7.8	5.56	0.573	10.3
	E 7	6.20	0.504	8.1	5.10	0.759	14.8
Shaking	N 28	9.02	1.289	14.2	7.66	2.843	37.1
	E 7	9.18	1.995	21.7	7.83	2.248	28.7

Initial glucose concentration is 9.51%.
* These cultures were analysed after 15~20 days' incubation at 30°.

TABLE II. GLYCEROL PRODUCTION BY VARIOUS STRAINS OF *Sacch. rouxii* IN THE 18% NaCl MEDIUM WITH AERATION

Strains	% of glucose consumed	% of crude glycerol produced	yield of glycerol on glucose consumed	Strains	% of glucose consumed	% of crude glycerol produced	yield of glycerol on glucose consumed
m 1	6.39	2.182	34.1%	R 5	3.03	0.761	25.1%
m 2	2.10	0.503	24.0	R 6	6.53	2.256	34.5
m 3	8.25	3.588	43.4	N 5	6.07	2.062	34.0
m 4	3.44	0.983	28.6	N 8	6.26	2.013	32.1
m 11	3.24	0.837	25.8	N 16	1.29	0.374	28.9
s 2	2.83	0.765	27.0	N 22	3.37	0.841	25.0
s 5	7.52	2.616	34.8	N 28	6.63	2.399	36.1
s 7	2.83	0.825	29.2	A 31	6.86	1.816	26.5
s 8	6.73	1.902	28.2	A 34	3.86	0.936	24.2
s 9	4.92	1.758	35.7	H 1	1.56	0.487	31.2
sal 1	1.84	0.415	22.6	H 4	1.49	0.405	27.2
sal 2	4.05	0.942	23.3	H 5	1.42	0.418	29.4
sal 4	1.15	0.310	26.9	H 7	5.75	1.348	23.4
j 3	5.43	1.663	30.6	E 7	8.22	2.537	30.8
R 4	2.83	0.755	26.6				

Initial glucose concentration is 10.0%.

75% *n*-butanol saturated with water as a developing solvent[7] and analysed by the above procedure of periodic oxidation.

Paper chromatograms were developed with a solvent composed of *n*-butanol, ethanol and water (4 : 1 : 5 by volume) according to the method of Hackman[8], and glycerol and similar compounds were detected by the method of Yoda[9]. The dried chromatogram was first sprayed with 0.5% aqueous KIO$_4$. After 1~2 minutes, the paper was sprayed with the following reagents: 15% aqueous MnSO$_4$: saturated *p-p'*-tetramethyl diaminodiphenylmethane in 2N acetic acid=1 : 1 v/v. White spots on the blue background indicate the locations of α-glycols.

EXPERIMENTAL AND RESULTS

(1) Effect of Aeration.

Glycerol production by two strains of *Sacch. rouxii* when they were cultured aerobically and anaerobically

---

8) R. H. Hackman and V. M. Trikojus, *Biochem. J.*, **51**, 653 (1952).

9) A. Yoda, *J. Chem. Soc. Japan*, **73**, 18 (1952).

in two media i.e. NaCl-free and 18% NaCl was examined. The results are shown in Table I. In the NaCl-free medium, the yield of glycerol in the anaerobic culture was 7∼8% on glucose consumed, and this yield was increased up to 14∼20% with aeration. In the 18% NaCl medium, as much as 30∼40% of glucose consumed was converted to glycerol in the aerobic conditions.

(2) Selection of an Excellent Strain for Glycerol Production.

In order to select the excellent strain, the glycerol production in the 18% NaCl medium by 29 strains of *Sacch. rouxii* which were isolated from soy-mashes and miso-pastes, was examined. As shown in Table II, the rate of fermentation and the yield of glycerol varied with the strains but in general, a high glycerol yield ranging from 22.6∼43.4% on sugar consumed was obtained by all the strains tested. Among these, the strain m 3 was found to be the most excellent strain for this production.

(3) Effects of Nitrogen Sources and Concentrations of Salts.

Employing strain m 3, the effects of various nitrogen sources and the concentration of NaCl or KCl on the glycerol production were examined. As nitrogen source, each of ammonium sulfate, ammonium chloride, urea, polypeptone, beef extract, ammonium lactate and ammonium acetate was added to the basal medium in place of casamino acid. The amounts of these nitrogen sources added are shown in Table III. In the media containing $(NH_4)_2SO_4$ or NH_4Cl, K-citrate-citric acid buffer was also added according to the reason previously reported[1]. Although the rate of fermentation differed in each medium with different nitrogen sources, very high yield of glycerol such as from 43∼50% on glucose consumed was obtained in all cases. The yield of glycerol was remarkably increased with increasing concentration of NaCl or KCl (Table III).

(4) Paper Chromatogram of the Fermentation Broth.

In the determination of glycerol, 2–3 butanediol and acetoin did not interfere, and the interfering effect of glucose was eliminated by correction. However, other polyhydric alcohols such as mannitol and erythritol would interfere to a great extent if they were present. Detection of other polyhydric alcohols in the fermentation broth of 18% NaCl (Table II) was carried out by means of paper chromatography. The result are illustrated in Fig. 1 and Photograph 1. These indicate that glycerol is the main product by aerobic fermentation in a high saline medium. Other polyhydric alcohols could not be obviously detected.

(5) Determination of True Glycerol by Partition Chromatography.

It was first examined whether or not partition chromatography by Neish[7] is applicable to the high saline solution. Solutions of 18% NaCl containing glycerol, mannitol, glucose, ethanediol and/or 1–2 propanediol, were prepared and diluted to a suitable concentration and then analyzed by partition chromatography after Neish[7]. As shown in Table IV, glycerol, sugar alcohol,

TABLE III. EFFECTS OF VARIOUS NITROGEN SOURCES AND THE CONCENTRATIONS OF SODIUM CHLORIDE AND POTASSIUM CHLORIDE

Media	% of glucose consumed	% of crude glycerol produced	yield of glycerol on glucose consumed
Basal medium	8.72	0.929	10.6%
〃 〃 +NaCl 6%	8.79	2.580	29.3
〃 〃 +NaCl 12%	8.57	2.195	25.6
〃 〃 +NaCl 18%	8.43	3.630	43.1
〃 〃 +KCl 11%	8.72	2.970	34.0
〃 〃 +KCl 22%	8.65	3.765	43.5
〃 〃 +NaCl 18%+Polypeptone 1%	8.39	3.018	36.0
〃 〃 + 〃 〃 +$(NH_4)_2SO_4$ 0.2%	6.85	3.252	47.4
〃 〃 + 〃 〃 +NH_4Cl 0.2%	3.48	1.746	50.1
〃 〃 + 〃 〃 +Urea 0.02%	4.99	2.533	50.7
〃 〃 + 〃 〃 +Ammonium lactate 0.2%	6.83	3.248	47.5
〃 〃 + 〃 〃 +Ammonium acetate 0.2%	6.55	3.229	49.2
〃 〃 + 〃 〃 +Beef extract 0.5%	8.20	3.589	43.8

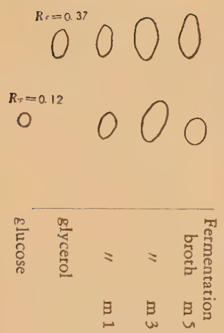
Initial glucose concentration is 10.65%.

TABLE IV. DETERMINATION OF GLYCEROL BY PARTITION CHROMATOGRAPHY

Solutions of glycerol, α-glycols, sugar, and fermentation broth	Values before developing*	Values after developing* Fractions			
		I	II	III	IV
glycerol	4.37 mg	0	0	0	4.37 mg
mannitol	3.92 mg	0	0	0	0
glucose	0.20 mg	0	0	0	0
mixture of glycerol, mannitol and glucose	4.22 mg	0	0	0	3.12 mg
ethanediol	3.01 mg	0	0	3.01 mg	0
1-2 propanediol	2.90 mg	0	0	3.00 mg	0
18% NaCl fermentation broth	3.85 mg	0	0	0	3.25 mg

* This values are all indicated as glycerol.

FIG. 1. Paper chromatogram of the fermented broth.



Photograph 1. Paper chromatogram of the fermented broth.

sugar, 1-2 propanediol and ethanediol were separated each other with satisfactory recovery. From the results of true glycerol determination in the 18% NaCl fermentation broth by this partition chromatography, 85% of the crude glycerol determined was found to be true glycerol. The detection of other compounds such as sugar alcohols which might be formed together with glycerol, and the results will be reported in the following paper.

DISCUSSIONS AND CONCLUSION

It is well-known that yeasts produce large quantities of glycerol in the presence of sulfite[10] or alkali[11]. However, fermentation processes without the addition of these agents may be commercially adventageous. Therefore, glycerol production by a normal fermentation process has recently been investigated by several workers: Nickerson and Carrol[12] found that *Zygosaccharomyces acidifaciens* produces glycerol in a yield of about 20% on the weight of sugar fermented in a normal fermentation process. Neish, Ledingham and Blackwood[13] showed that another type of fermentation by *Bacillus licheniformis* leads to glycerol production together with 2-3 butanediol, lactic acid, ethanol and formic acid. Spencer and Sallans[14] reported that osmophilic yeasts isolated from fermenting honey and other sources produce one or more of the sugar alcohols, as glycerol, erythritol, D-arabitol, mannitol and two unidentified polyhydric alcohols. As much as 60% of the glucose dissimilated was converted to polyhydric alcohols. Fukui and Mohara[15] studied on the formation of glycerol by the use of sulfathiazole as an antagonist against yeast cocarboxylase. For example, when 0.8 g of sulfathiazole was added to 100 ml of the medium and the medium was fermented by 2 g of pressed yeast, 3.4 g of glycerol and 2.6 g of ethylalcohol were formed from 10 g of glucose after 7 days at 30°.

---

10) C. Neuberg and E. Reinfurth, *Biochem. Z.*, **92**, 234 (1918); *Ber. Deut. Chem. Ges.*, **52**, 1677 (1919).

11) C. Neuberg and E. Farber, *Biochem. Z.*, **78**, 238 (1917).

12) W. J. Nickerson and W. R. Carrol, *Arch. Biochem.*, **7**, 257 (1945).

13) A. C. Neish, G. A. Ledingham and A. C. Blackwood, *Can. J. Research.*, **23B**, 290 (1945).

14) J.F.T. Spencer and H. R. Sallans, *Can. J. Microbiol.*, **2**, 72 (1956).

15) S. Fukui and K. Mohara, *J. Fermentation Technol.*, **29**, 198 (1951).

In the present paper, it has been reported that yeasts produce much higher yield of glycerol (as much as 40~50% on sugar fermented) in a high saline medium under aerobic condititon, although a small amount is formed in the ordinary plain medium. This fact is of special interest not only that sodium chloride affects the yeast metabolism to alter the fermentation pathway but also that the industrial process of glycerol production is proved to be promising in view of its high yield and simplicity in procedure as compard with those by the use of steering agents.

Acknowledgement The author wishes to express his sincerest thanks to Emeritus Prof. K. Sakaguchi of University of Tokyo for his kind guidance and encouragement throughout this work. Great indebtness is also acknowledged to Prof. T. Asai and Prof. Y. Sumiki for their valuable suggestions. Thanks are also due to Dr. M. Mogi, the Director of Noda Institute for Scientific Research, for his encouragement and to Mr. A. Kaneko for his willing help at all stages of this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 363~369, 1959]

Studies on the Proteolytic Enzymes of Black Aspergilli

Part V. The Specificity of Crystalline *Asp. Saitoi* Proteinase by Application of the Sanger DNP-Method

By Fumihiko Yoshida, Michitaro Nagasawa and Eiji Ichishima

Noda Institute for Scientific Research

Received November 22, 1958

The specificity of crystalline *Asp. Saitoi* proteinase on oxidized lysozyme has been investigated by application of the Sanger DNP-method.

It was found that this proteinase has a much broader specificity as compared with pepsin and *Bac. subtilis* proteinase.

Details of the specificity of well-known proteolytic enzymes have been investigated employing synthetic peptides and their derivatives[1].

However, at present, it is also possible to use proteins as a substrate for this purpose adopting suitable techniques for end-group analysis studies in peptides and proteins according to the Sanger DNP-method[2,3,4]. For this reason, horse globin and ovalbumin[5] have been used for the determination of specificity of certain proteolytic enzymes.

In the previous paper of this series, the specificity of *Asp. Saitoi* proteinase on synthetic substrates was investigated, and it was found that the enzyme had much broader specificity than pepsin[6]. In this paper, a study has been conducted to determine the specificity of this enzyme through analysis of free amino acids and N-terminal amino acid residues of peptides liberated from oxidized lysozyme by the enzymatic digestion. The abbreviation for amino acid residues as suggested by Brand and Edsall⁷⁾ are adopted here.

---

[1] J. Fruton and M. Bergmann, *J. Biol. Chem.*, **127**, 627 (1939).

[2] F. Sanger, *Biochem. J.*, **39**, 507 (1949).

[3] F. Sanger and H. Tuppy, *ibid.*, **49**, 481 (1951).

[4] F. Sanger and E.O.P. Thompson, *ibid.*, **53**, 336 (1953).

[5] P. Duesnuelle et al., *Biochim. Biophys. Acta*, **5**, 116 (1950).

[6] F. Yoshida and M. Nagasawa, This Bulletin, **20**, 262 (1956).

EXPERIMENTAL

1) **Standard DNP-amino acids:** Standard DNP-amino acids were supplied by the Wakō Pure Chemical Co., Ltd. For the paper chromatographic analysis, the ether soluble DNP-amino acids were dissolved in acetone to M/100 solutions, and the water soluble ones were dissolved in methylethylketone containing a small amount of hydrochloric acid.

2) **Paper chromatography:** The chromatagraphic separation of DNP-amino acids was effected two dimensionally on a buffered filter paper (40×40 cm), Tōyō Roshi No. 51, which had been previously sprayed with M/10 phthalate buffer (pH 6.0) and dried at room temperature. Many of the solvent systems tried proved to be unsuitable, because the DNP-amino acids ran fast near the solvent front or else somewhat 'tailed'. The solvent systems which can be successfully used are as follows:

Ether soluble fraction
1. tert-AmOH-phthalate buffer (pH 6.0)⁸⁾
2. 1.5 M phosphate buffer (pH 6.0)⁹⁾

Water soluble fraction
1. *n*-BuOH-1% NH_3 (1/1, v/v)¹⁰⁾
2. 0.75 M phosphate buffer (pH 6.0)¹¹⁾

3) **Crystalline *Asp. Saitoi* proteinase:** A crystalline *Asp. Saitoi* proteinase was prepared according to the method of Yoshida¹²⁾.

4) **Substrate** (Oxidation of lysozyme with performic acid¹³⁾): Five ml of 30% hydrogen peroxide (R.G.) was added to 45 ml of 90% formic acid and after standing at room temperature for 1 hr., the mixture was cooled down to −8° in a freezer. The performic acid mixture was added to a cold solution of 1 gr of lysozyme (supplied by N.B.C.) in 10 ml of 90% formic acid. After being left for 30 min. at −8° (or at the same temperature) the reaction mixture was diluted with 900 ml of ice water, the diluted mixture immediately frozen with ethanol-dry ice and the solvents were removed by lyophilization. The residue was dissolved in 50 ml of water and lyophilized again.

5) **Enzymatic hydrolysis of substrate:** Thirty mg. of oxidized lysozyme dissolved in 3 ml of dilute hydrochloric acid solution (pH 2.7) was mixed with 0.5 ml of enzyme solution containing 0.3 mg of *Asp. Saitoi* proteinase and allowed to react at 40° for 48 hours. After boiling to inactivate the enzyme, the solution was lyophilized. As a control experiment, 3 ml of oxidized lysozyme solutuion was mixed with 0.5 ml of the previously boiled enzyme solution and the mixture was treated as above.

6) **Reaction with 2,4-dinitrofluorobenzene:** Since it was recognized that the enzyme splitted protein molecules to samller fragments that pepsin¹⁴⁾, the scheme shown in Fig. 1 has been used to determine free amino acids and N-terminal residues in hydrolyzate. In order to identify the N-terminal residues of both undigested proteins and digested products, the method of Okunuki et al.¹⁵⁾ was applied.

Thirty mg of hydrolyzate was dissolved in 3 ml of water, and 30 mg of sodium bicarbonate was added; the solution was adjusted to pH 8.5 by 0.8 M sodium bicarbonate buffer. Then, the buffered solution was mixed with 10 ml of alcoholic solution of 2,4-dinitrofluorobenzen (DNFB) (0.1 ml DNFB for the control, 0.5 ml DNFB for 48 hour's digestant) and the resulting mixture was shaken for 3 hr. at room temperature. After removing ethanol in vacuo, 5 ml of water was added and the excess DNFB was extracted with ether. Ether was removed from the aqueous layer in vacuo and the solution was neutralizated with a few drops of conc. hydrochloric acid. After the addition of an equal volume of conc. hydrochloric acid to the solution, the mixture was hydrolyzed in a sealed tube at 115~120° for 12 hr.

On the other hand, the samples from which the excess DNFB and DNA (dinitroaniline) were removed were acidified with conc. hydrochloric acid and extracted with ether to determine free amino acids and their derivatives. DNP-amino acids were extracted with ether and most of dinitrophenol (DNP) was removed according to the method of Mills¹⁶⁾. The aqueous phase was than evaporated in vacuo and extracted with methylethylketone.

---

7) E. Brand et al., *Ann. Rev. Biochem.*, **16**, 224 (1947).

8) S. Blackburn and A.G. Lowther, *Biochem. J.*, **48**, 126 (1951).

9) A. L. Levy, *Nature*, **174**, 126 (1954).

10) G. Braunitzer, *Ber.*, **88**, 2025 (1955).

11) J. W. Davies and G. Harris, *Arch. Biochem. Biophys.*, **74**, 229 (1958).

12) F. Yoshida and M. Nagasawa, This Bulletin, **22**, 32 (1958).

13) G. Toennis and R. P. Homiller, *J. Am. Chem. Soc.*, **64**, 3054 (1942).

14) F. Yoshida and M. Nagasawa, *Proc. Intern. Symposium Enzyme Chem.*, **2**, 504 (1957).

15) K. Okunuki et al., *J. Biochem. Japan.*, **43**, 857 (1956).

16) G. L. Mills, *Biochem. J.*, **50**, 707 (1952).

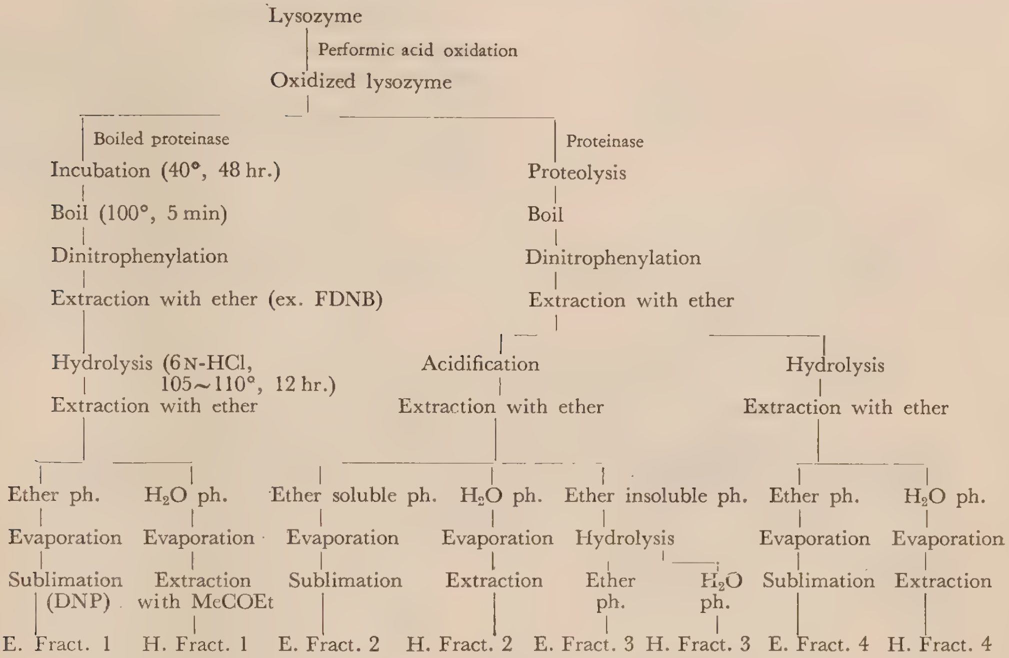


FIG. I. Procedure for the Determination of N-Terminal Residues

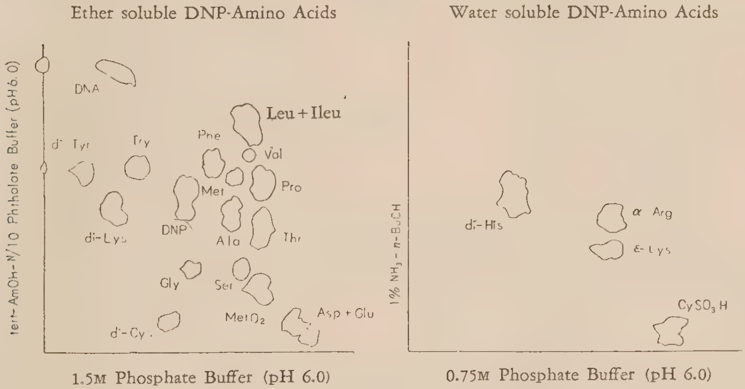


FIG. 2. Paperchromatogram of Standard DNP-Amino Acids

TABLE I. RECOVERY OF STANDARD DNP-AMINO ACIDS ON PAPERCHROMATOGRAPHY (2-Dimensional)

DNP-Amino Acids	Photometric Solvents	Recovery (%)	DNP-Amino Acids	Photometric Solvents	Recovery (%)
Ala	1%-$NaHCO_3$	61	Thr	1%-$NaHCO_3$	76
Asp+Glu	//	73	di-Tyr	//	87
Gly	//	84	Try	//	80
Leu+Ileu	//	84	Val	//	85
di-Lys	Glacial AcOH	94			
Met	1%-$NaHCO_3$	69	α-Arg	1 N-HCl	87
Phe	//	67	$CySO_3H$	//	74
Pro	//	70	di-His	//	83
Ser	//	89	ε-Lys	//	91

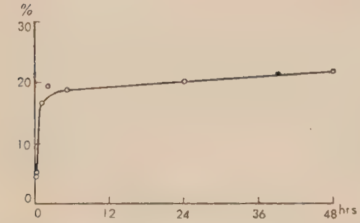


FIG. 3. Digestion Curve of Oxidized Lysozyme by *Asp. Saitoi* Proteinase

7) **Quantitative analysis**[9]**:** DNA-amino acids were separated with paper chromatography using the solvents mentioned previously. Each spot was eluted with suitable solvent systems and the amount of each DNP-amino acid was determined spectrophotometrically at 360 mμ (for most of DNP-amino acids), 355 mμ (for di-DNP-Lys), 380 mμ (for DNP-Pro), respectively.

RESULTS AND DISCUSSION

In general, the separation of DNP-amino acids on paper chromatogram has resulted in only a

TABLE II. N-TERMINAL RESIDUES IN DIGESTION PRODUCT FROM OXIDIZED LYSOZYME BY THE ACTION OF *Asp. Saitoi* PROTEINASE

DNP-Amino Acids	Moles of residue per molecular of oxidized lysozyme*	Moles of residues detected as N-Terminal**				
		Fract. 1 (Control)		Fract. 2	Fract. 3	Fract. 4
Ala	10−12			0.76	0.47	3.21
Asp+Glu	20+5	0.32		2.28	0.47	6.18
Gly	11−12		Gly..1	0.66		3.18
			..2	0.65		
			..3	0.43		
di-Lys	1 (+5)	0.465		1.06	0.49	0.75
Leu+Ileu	8+6		Leu+..1	1.70		4.10
			Ileu..2	1.54		
Phe	3			0.26		1.18
Pro	2			0.84		0.78
Ser	9	0.05	Ser..1	0.33	0.19	1.19
			..2	0.18		
Thr	7			0.21		1.71
di-Tyr	3			0.67	0.34	0.67
Val	5−6	0.07		0.39	0.33	0.99
Unknown 1.E				2.44		0.40
2.E						0.86
α-Arg	11					1.67
$CySO_3H$	10			3.06	1.37	3.92
di-His	1					0.66
ε-Lys	(Initial 5)	2.90		1.50	1.57	3.16
Unknown 1.H		+		0.14		0.17
2.H				0.26	0.29	0.19
3.H				0.87	0.44	0.26
4.H				0.44		
5.H				0.30		
6.H				0.58		
7.H				0.18		0.57
8.H				0.19		+
9.H						0.67

* Amino acids composition of oxidized lysozyme was assumed according to Thompson.[17]

** Recovery factor of hydrolysis were adopted according to the literature.[2,18,19]

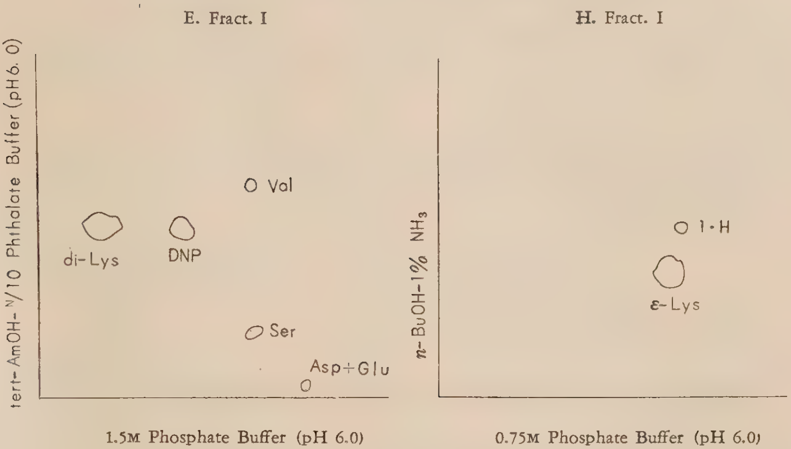


FIG. 4-A. Paperchromatogram of N-Terminal Residues in Digestion Product from Oxidized Lysozyme by the Action of *Asp. Saitoi* Proteinase

The abbreviation of E. and H. indicated unknown DNP-derivatives in the ether soluble phase, water soluble phase respectively.

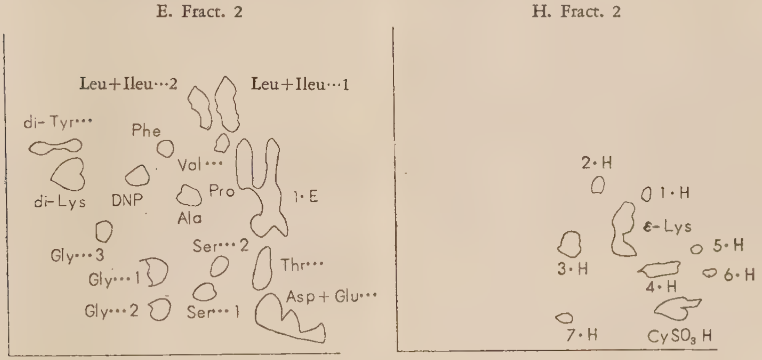


FIG. 4-B

The abbreviation Thr..., etc. indicated the DNP-peptide, which corresponded to DNP-Thr on paperchromatogram.

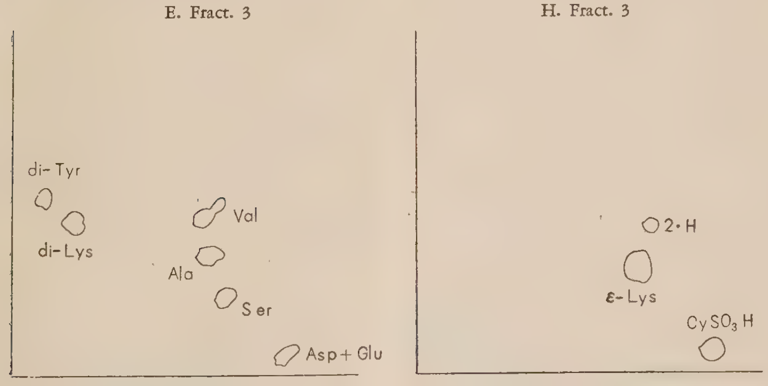
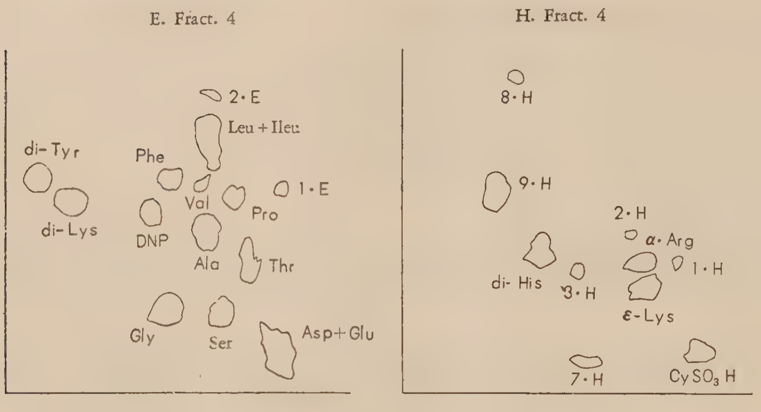


FIG. 4-C


E. Fract. 4

H. Fract. 4

FIG. 4–D

limited success due to the 'tailing' of spots. Blackburn et al.[8] observed that DNP-amino acids might be successfully separated on buffered one-dimensional paper chromatograms, compact spots without 'tailing' which possess characteristic rates (R_F value) being formed in suitable solvents. Levy[9] established a method for the quantitative microanalysis of DNP-amino acid mixture on a two-dimensional filter paper chromatogram, which is followed by quantitative elution from the paper and estimation at 360 mμ.

The standard DNP-amino acids chromatogram and their recoveries on the paper chromatogram are shown in Fig. 2 and Table I, respectively.

The course of hydrolysis of oxidized lysozyme by *Asp. Saitoi* proteinase is shown in Fig. 3.

The results of analysis by the Sanger DNP-method are summarized in Fig. 4-A to 4-D and Table II. As shown in Fig. 4-A very small quantities of DNP-derivatives of Val, Ser, and Asp+Glu appearing on the chromatogram may represent impurities in the preparation of terminal groups resulting from partial breakdown of peptide chain of the substrate by performic acid oxidation. From Fig. 4-B and Fig. 4-D, it was recognized that Ala, Asp +Glu, Gly, Leu+Ileu, Lys, Phe, Pro, Ser, Thr, Tyr, Val, Arg, $CySO_3H$, and His are found as N-terminal residues of peptides resulting from oxidized lysozyme by the action of *Asp. Saitoi* proteinase. In comparison with N-terminal residues in the digestion products with pepsin[20] and *Bac. subtilis* proteinase[15], the mold proteinase appears to have a considerably wider range of specificity than the other two enzymes.

In recent years, though the structure of lysozyme has been studied by Thompson[17,21], Fromageot and Jolles[22], and others, some amino acid sequences are still obscure.

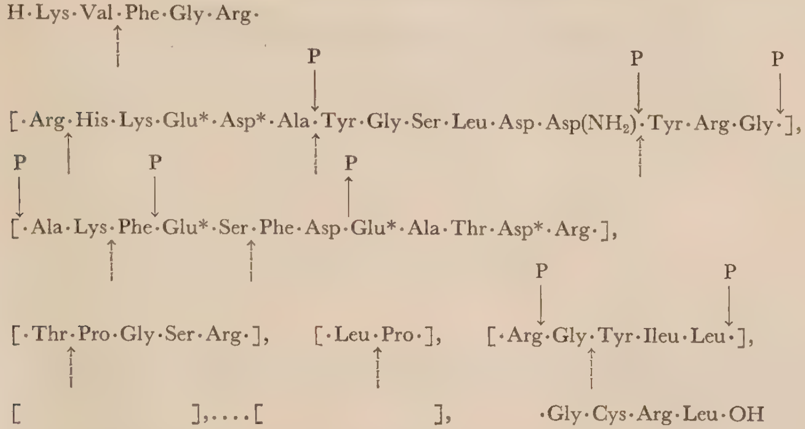
The main site of lysozyme molecule at which splitting occurrs under the action of *Asp. Saitoi* proteinase and pepsin, in concern of His, Phe, Pro and Tyr residues are indicated in Table III. Regarding the N-terminal residues other than the above four residues, it is impossible to determine which bonds are splitted by the enzyme, because the number of these amino acids contained in a lysozyme molecule is too large.

However, because of its broader specificity, *Asp. Saitoi* proteinase could liberate a variety of unsplitted peptides as demonstrated in Fig. 4-B. Therefore, it is expected that the specificity of *Asp. Saitoi* proteinase should be further clarified by the determination of amino acid sequences of these unsplitted peptides. Such

---

[17] A. R. Thompson, *Biochem. J.*, **60**, 507 (1955).
[18] W. A. Schroeder, *J. Am. Chem. Soc.*, **75**, 4612 (1952).
[19] R. R. Porter and F. Sanger, *Biochem. J.*, **42**, 287 (1948).
[20] P. Jolles et al., *Biochim. Biophys. Acta.*, **27**, 439 (1958).
[21] A. R. Thompson, *Biochem. J.*, **61**, 253 (1955).
[22] C. Fromageot and P. Jolles, *Proc. Intern. Symposium Enzyme Chem.*, **2**, 504 (1957).

TABLE III. SITE OF CLEAVAGE OF OXIDIZED LYSOZYME BY *Asp. Saitoi* PROTEINASE


H·Lys·Val·Phe·Gly·Arg·
P
P
P
[·Arg·His·Lys·Glu*·Asp*·Ala·Tyr·Gly·Ser·Leu·Asp·Asp(NH₂)·Tyr·Arg·Gly·],
P
P
P
[·Ala·Lys·Phe·Glu*·Ser·Phe·Asp·Glu*·Ala·Thr·Asp*·Arg·],
P
P
[·Thr·Pro·Gly·Ser·Arg·],
[·Leu·Pro·],
[·Arg·Gly·Tyr·Ileu·Leu·],
[ ],....[ ],
·Gly·Cys·Arg·Leu·OH

←— Bonds split by *Asp. Saitoi* Proteinase.
←--- Some of these bonds split by *Asp. Saitoi* proteinase.
←—P Bonds split by pepsin.
* It has not been determined whether or not these residues contain an amide group.

attempts are now under way.

SUMMARY

1. The oxidized egg-white lysozyme was hydrolyzed with crystalline *Asp. Saitoi* proteinase and the resulting N-terminal residues were detected by the DNP-technique of Sanger.

2. It was proved that *Asp. Saitoi* proteinase has a considerably wider range of specificity than pepsin and *Bac. subtilis* proteinase.

Acknowlegement We wish to express our thanks to Dr. K. Sakaguchi, Dr. T. Asai and Dr. B. Maruo, University of Tokyo, for their constant guidance and also to Dr. M. Mogi for his support in this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 370~376, 1959]

Studies on Amino Acids XII.

Studies on the Enzymatic Resolution (X): Acylase Activity in Plants

By Ichiro CHIBATA and Tetsuya TOSA

*Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd.**

Received November 28, 1958

Although acylase activities in animal tissues, molds, bacteria and yeast have been clarified, nothing has been reported on the acylase activity in plants. Therefore, as a part of a series of studies intending to elucidate the biological significance of the presence of acylase activity, activity in plants was investigated.

As a result, the occurrence of acylase activity in plants such as vegetables, potatoes, pulses, cereals and fruits, has been confirmed for the first time. Besides the above higher plants a relatively higher acylase activity was also found in mushroom.

The authors have reported on the distribution and enzymatic properties of acylase activity, that is, hydrolytic activities towards acyl amino acids, in molds[1,2], bacteria[3] and yeast[4], and also on its application for the resolution of amino acids[5~8].

In succession to these previous studies, the results of investigation on the acylase activity in plants are presented here. Since any available report on the acylase activity in plants has not yet appeared, the authors prepared enzyme solutions from several vegetables, fruits, cereals, pulses and potatoes and have studied their hydrolytic activities towards acyl amino acids. As a result, the occurrence of acylase activity in plants has been confirmed for the first time.

Besides higher plants, acylase activity in mushroom was also investigated for the purpose of comparative study.

MATERIALS AND METHODS

The preparation of amino acid derivatives such as acetyl-DL-methionine, chloroacetyl-DL-phenylalanine, acetyl-L-glutamic acid, ε-benzoyl-α-acetyl-DL-lysine and glycyl-L-methionine, has been reported by the authors in previous papers.

Since procedures for the preparation of enzyme solutions somewhat varied according to the plants employed, the procedures are described in respect of the kind of the plants tested.

Standard enzyme assay was carried out by incubating a mixture of 1 ml of enzyme solution, 1 ml of 0.025 M substrate solution (0.05 M for racemic compounds) and 1 ml of phosphate buffer (M/15, pH 5.5, 7.0 and 8.0) at 37° for 6 hours, unless otherwise stated. Liberated amino acids were measured by the colorimetric ninhydrin method. Enzymatic activity was expressed in terms of μM of liberated amino acid per hour per mg of protein N or per g of fresh matter. Protein N was determined by the micro-Kjeldahl method of the precipitate obtained from the enzyme treated with 10% trichloroacetic acid.

RESULTS

Acylase Activity of Vegetables

Radish, carrot, cabbage, spinach and pumpkin (*Cucurbita pepo* and *Cucurbita moschata*) were chosen as the vegetable samples.

---

* Kashima-cho, Higashi Yodogawa-ku, Osaka.

[1] I. Chibata, T. Ishikawa and S. Yamada, This Bulletin, **21**, 300 (1957).

[2] I. Chibata, T. Ishikawa and S. Yamada, This Bulletin, **21**, 304 (1957).

[3] I. Chibata, M. Kisumi and S. Yamada, This Bulletin, **22**, 24 (1958).

[4] I. Chibata and T. Ishikawa, This Bulletin, **22**, 218 (1958).

[5] S. Yamada, I. Chibata and S. Yamada, *J. Pharm. Soc. Japan*, **75**, 113 (1955).

[6] I. Chibata, S. Yamada and S. Yamada, This Bulletin, **20**, 174 (1956).

[7] I. Chibata, A. Watanabe and S. Yamada, This Bulletin, **21**, 291 (1957).

[8] I. Chibata, A. Watanabe and S. Yamada, This Bulletin, **21**, 296 (1957).

TABLE I. ACYLASE ACTIVITY IN VEGETABLES

Plants	Substrates	Acetyl-methionine		Chloroacetyl-phenylalanine		Acetyl-glutamic acid		ε-Benzoyl-α-acetyllysine		Glycyl-methionine	
	Activity \ pH	7.0	5.5	7.0	5.5	7.0	5.5	7.0	5.5	7.0	8.0
Radish	μM/hr/N mg of protein	21.21	5.85	10.71	6.32	0	0	0	0	74.00	74.00
	μM/hr/g of fresh matter	0.08	0.02	0.04	0.02	0	0	0	0	0.27	0.27
Carrot	μM/hr/N mg of protein	3.48	0.74	0	0	0	0	0	0	3.08	2.11
	μM/hr/g of fresh matter	0.26	0.06	0	0	0	0	0	0	0.23	0.16
Cabbage	μM/hr/N mg of protein	5.14	3.48	0	0	0	0	0	0	3.85	3.02
	μM/hr/g of fresh matter	0.10	0.07	0	0	0	0	0	0	0.07	0.06
Spinach	μM/hr/N mg of protein	2.74	2.84	0.28	0.31	0.41	0.78	0	0	2.31	1.24
	μM/hr/g of fresh matter	0.18	0.19	0.02	0.02	0.03	0.05	0	0	0.15	0.08
Pumpkin (*Cucurbita pepo*)	μM/hr/N mg of protein	6.53	5.75	1.37	0	0	0	0	0	4.84	4.84
	μM/hr/g of fresh matter	1.17	1.03	0.24	0	0	0	0	0	0.87	0.87
Pumpkin (*Cucurbita moschata*)	μM/hr/N mg of protein	3.64	5.12	1.67	0	0.47	0.85	0	0	5.63	5.40
	μM/hr/g of fresh matter	0.30	0.42	0.14	0	0.04	0.07	0	0	0.46	0.44

The vegetables were chopped and homogenized for ten minutes in a Waring blendor with a phosphate buffer (M/50, pH 7.2) of 1/2∼1/4 weight of the fresh matter. After standing in a cool place for 1 hour, the homogenized mixture was filtered with a gauze, and the filtrate was further centrifuged. To the supernatant solution kept below −10°, acetone (70% by volume), previously chilled to −10∼−15°, was added with stirring. The precipitate was collected by centrifugation and dissolved in the phosphate buffer M/50, pH 7.2. The solution was dialyzed overnight against distilled water in a cool place. The precipitate formed during dialysis was removed by centrifugation, and the supernatant solution was employed as the enzyme solution.

In cases of cabbage and spinach, before the acetone treatment described in the above procedure, the supernatant solution was made to pH 5.2 with acetic acid and centrifuged to remove chloroplasts. The supernatant solution thus obtained was made up to pH 7.0 with sodium bicarbonate and treated with acetone after the above procedure.

With these enzyme preparations obtained from vegetables, hydrolytic activities to several acyl amino acids and dipeptide were investigated.

As shown in Table I, it was revealed that acylase activity occurs widely in vegetables and that the highest activity was shown in case of radish for the unit of protein N and in pumpkin (*Cucurbita pepo*) for the unit of fresh matter. Although acetylmethionine could be hydrolyzed by the enzyme solutions from cabbage and carrot, other acyl amino acids were not susceptible to these enzyme preparations, and ε-benzoyl-α-acetyllysine was not hydrolyzed by all of the enzyme solutions tested. Also, it was found that peptidase activity towards glycylmethionine occures in all of these vegetables.

Acylase Activity of Fruits

Mandarin orange, banana and fig were chosen in order to investigate acylase activity in fruits. In cases of mandarin orange and banana, activities in both the peel portion and the edible portion were examined.

The enzyme solution was prepared by extraction with phosphate buffer and acetone precipitation according to the method described in the case of vegetables.

As shown in Table II, the peel portion of mandarin orange and fig, though slightly, showed hydrolytic

TABLE II. ACYLASE ACTIVITY OF FRUITS

Plants		Substrates / Activity	Acetyl-methionine		Chloroacetyl-phenylalanine		Acetyl-glutamic acid		ε-Benzoyl-α-acetyllysine		Glycyl-methionine	
		pH	7.0	5.5	7.0	5.5	7.0	5.5	7.0	5.5	7.0	8.0
Mandarin orange	(Peel portion)	μM/hr/N mg of protein	1.68	1.32	0.18	0.06	0	0.09	0	0	1.19	0.64
		μM/hr/g of fresh matter	0.28	0.22	0.03	0.01	0	0.02	0	0	0.20	0.11
	(Edible portion)	μM/hr/N mg of protein	0	0	0	0	0	0	0	0	0	0
		μM/hr/g of fresh matter	0	0	0	0	0	0	0	0	0	0
Banana	(Peel portion)	μM/hr/N mg of protein	0	0	0	0	0	0	0	0	0	0
		μM/hr/g of fresh matter	0	0	0	0	0	0	0	0	0	0
	(Edible portion)	μM/hr/N mg of protein	0	0	0	0	0	0	0	0	0	0
		μM/hr/g of fresh matter	0	0	0	0	0	0	0	0	0	0
Fig		μM/hr/N mg of protein	0.35	0	0.14	0.41	0.31	0.45	0	0	0	0
		μM/hr/g of fresh matter	0.01	0	<0.01	0.01	0.01	0.01	0	0	0	0

activities, although, any hydrolytic activities were not found either in banana or in the edible portion of mandarin orange.

Acylase Activity in Cereals and Pulses

For cereals and pulses, the following rice, wheat, barley, peas and soy-bean were chosen, and hydrolytic activities were measured in both states of resting and seedling. In case of peas, mature resting peas and immature peas in the pod were investigated.

Resting seeds were pulverlized and extracted for 30 minutes under stirring with phosphate buffer (M/50, pH 7.2) weighing twice as much as the fresh matter. After standing for 1.5 hours in a cool place, the mixture was filtered with a gauze and the filtrate was centrifuged. The supernatant solution was treated with acetone and the enzyme solution was prepared in accordance with the procedure previously described in case of vegetables.

For the enzyme preparation from seedlings, germination was carried out as follows: Resting seeds, previously soaked overnight in water, were transferred on a wet gauze and filterpaper layed in a petri dish. The dish was placed in an incubator and kept at 27° for germination. When average length of sprouts reached 2~4 cm, the seedlings were collected and weighed. The period required for germination was 4 days for rice and wheat, 5 days for barley and 2 days for soy-bean, respectively. From these seedlings, the enzyme solution was prepared in the same way as in case of vegetables. The preparation from immature peas was carried out as in case of spinach and cabbage in order to remove chloroplasts.

The results of the experiments on the hydrolytic activities of these enzyme preparations are shown in Tables III and IV. In these Tables, the activity for fresh matter of seedlings is expressed by converting the weight of seedlings to that of the resting seeds for convenience of comparison of activities in resting and germinating states.

The results disclosed that hydrolytic activities are widely distributed in both resting and germinating states of cereals and pulses. Although acetylglutamic acid was insusceptible to the enzyme preparations from resting state of wheat, barley and soy-bean, this substrate was hydrolyzed by preparations from seedlings. However this differed in case of rice, the enzyme solution from the resting seeds showed a slight activity towards acetylglutamic acid. ε-Benzoyl-α-acetyllysine was quite insusceptible to all preparations from cereals and pulses as in cases of other plants tested. Through these experiments, a tendency to show higher hydrolytic activities on germination than resting state was often observed, although it was not so evident.

TABLE III. ACYLASE ACTIVITY OF CEREALS

Plants		Substrates	Acetyl-methionine		Chloroacetyl-phenylalanine		Acetyl-glutamic acid		ε-Benzoyl-α-acetyllysine		Glycyl-methionine	
		Activity \ pH	7.0	5.5	7.0	5.5	7.0	5.5	7.0	5.5	7.0	8.0
Rice	(Resting)	μM/hr/N mg of protein	3.17	2.45	1.68	1.45	0.20	0	0	0	2.35	2.71
		μM/hr/g of fresh matter	0.74	0.56	0.39	0.34	0.05	0	0	0	0.55	0.63
	(Seedling)	μM/hr/N mg of protein	4.64	3.68	1.47	0.96	0	0	0	0	3.14	3.76
		μM/hr/g of fresh matter	1.44	1.14	0.45	0.30	0	0	0	0	0.97	1.16
Wheat	(Resting)	μM/hr/N mg of protein	1.52	0.74	1.21	1.20	0	0	0	0	2.55	2.28
		μM/hr/g of fresh matter	0.63	0.31	0.50	0.50	0	0	0	0	1.06	0.95
	(Seedling)	μM/hr/N mg of protein	1.68	0.94	0.95	1.41	0.49	0.12	0	0	6.90	5.99
		μM/hr/g of fresh matter	0.36	0.20	0.20	0.30	0.10	0.02	0	0	1.45	1.26
Barley	(Resting)	μM/hr/N mg of protein	1.04	0.97	1.15	0.29	0	0	0	0	2.30	1.99
		μM/hr/g of fresh matter	0.33	0.31	0.36	0.09	0	0	0	0	0.73	0.63
	(Seedling)	μM/hr/N mg of protein	0.44	0.31	0.36	0.30	0.15	0.25	0	0	0.62	0.61
		μM/hr/g of fresh matter	0.29	0.22	0.25	0.25	0.11	0.17	0	0	0.43	0.42

TABLE IV. ACYLASE ACTIVITY OF PULSES

Plants		Substrates	Acetyl-methionine		Chloroacetyl-phenylalanine		Acetyl-glutamic acid		ε-Benzoyl-α-acetyllysine		Glycyl-methionine	
		Activity \ pH	7.0	5.5	7.0	5.5	7.0	5.5	7.0	5.5	7.0	8.0
Pea	(Resting)	μM/hr/N mg of protein	1.12	1.03	0.72	1.30	0.17	0.84	0	0	0.63	0.88
		μM/hr/g of fresh matter	2.72	2.51	1.76	2.73	0.40	2.05	0	0	1.53	2.14
	(Immature)	μM/hr/N mg of protein	3.59	3.39	2.74	1.99	0.41	0.74	0	0	2.63	1.39
		μM/hr/g of fresh matter	1.03	0.97	0.79	0.57	0.12	0.21	0	0	0.76	0.40
Soy-bean	(Resting)	μM/hr/N mg of protein	0.26	0.26	0.10	0.09	0	0	0	0	0.40	0.46
		μM/hr/g of fresh matter	1.14	1.21	0.47	0.39	0	0	0	0	1.83	2.11
	(Seedling)	μM/hr/N mg of protein	0.57	0.12	0	0	0.36	0.10	0	0	0.11	0.11
		μM/hr/g of fresh matter	2.51	0.52	0	0	1.59	0.44	0	0	0.49	0.49

Table V. Acylase Activity of Potatoes

Plants	Substrates / Activity \ pH	Acetyl-methionine		Chloroacetyl-phenylalanine		Acetyl-glutamic acid		ε-Benzoyl-α-acetyllysine		Glycyl-methionine	
		7.0	5.5	7.0	5.5	7.0	5.5	7.0	5.5	7.0	8.0
Potato	μM/hr/N mg of protein	3.50	3.39	0.42	0.28	0.32	0.99	0	0	0	0
	μM/hr/g of fresh matter	0.64	0.62	0.08	0.05	0.06	0.18	0	0	0	0
Sweet potato	μM/hr/N mg of protein	0.43	0.31	0	0	0	0	0	0	0.10	0.10
	μM/hr/g of fresh matter	0.09	0.06	0	0	0	0	0	0	0.02	0.02

Table VI. Acylase Activity of Mushroom

Plant	Substrates / Activity \ pH	Acetyl-methionine		Chloroacetyl-phenylalanine		Acetyl-glutamic acid		ε-Benzoyl-α-acetyllysine		Glycyl-methionine	
		7.0	5.5	7.0	5.5	7.0	5.5	7.0	5.5	7.0	8.0
Mushroom "Matsutake" (*Armilaria edoides*)	μM/hr/N mg of protein	10.42	4.98	15.74	19.55	5.10	3.04	6.81	6.67	28.40	17.63
	μM/hr/g of fresh matter	4.48	2.14	6.77	8.42	2.19	1.31	2.93	2.86	12.21	7.58

Acylase Activity of Potatoes

Potato and sweet potato were selected for the study on hydrolytic activities of potatoes.

The enzyme solution from potatoes was prepared according to the method described for vegetables.

The results of the studies carried out with the above preparations are shown in Table V. Although acylase activity occurs in potato, peptidase action towards glycylmethionine could not be observed but in case of sweet potato, weak hydrolytic activities towards acetylmethionine and glycylmethionine were observed. Similar to other plants tested, ε-benzoyl-α-acetyllysine was insusceptible to these preparations from potatoes.

Acylase Activity of Mushroom

A mushroom, "Matsutake" (*Armilaria edoides*), was homogenized for 15 minutes in a Waring blendor with phosphate buffer (M/50, pH 7.2) weighing twice as much as the fresh matter. After standing for 2 hours in a cool place, the mixture was filtered and the filtrate was used as the enzyme solution.

The results shown in the Table VI, indicate the higher and somewhat different hydrolytic activities of mushroom from other higher plants previously tested.

DISCUSSION

Although a number of reports have appeared on the proteolytic enzymes in plants, no any report on the hydrolytic activities towards acyl amino acids has been available. Through the present study conducted by the authors, the occurrence of acylase activity in plants has been confirmed for the first time.

Since it was difficult to carry out the enzyme assay employing crude extracts of plants as the enzyme solution except in the case of mushroom, the enzyme solution prepared by procedures of extraction, acetone precipitation and following dialysis, were employed in these experiments. Therefore it was possible to assume that the loss of enzyme activities occurred during the preparation of enzyme solution and a somewhat lower enzymatic activity than the original state was indicated in the results obtained. However, these studies sufficiently interpreted the general tendency of acylase activities in plants.

Among the fruits tested, hydrolytic activities were not found in banana under the conditions employed by the authors. As the occurrence of several enzymes in banana has been previously known, from the result obtained it can not be easily decided whether banana originally lacks the acylase or that inadequate preparation procedure diminishes the enzyme activity.

In cases of cereals and pulses, hydrolytic activities in both resting and germinating states were comparatively studied. It has been well known that some enzyme activities in seeds markedly increase during the period of germination. Also, from experiments conducted by the authors, higher activities were often observed in the germinating state than resting seeds. However, the increase in acylase activity by germination was not so apparent and in few cases any activation was not observed.

This fact may be interpreted that in both resting and germinating states acylase activity plays a certain rôle that results in inconsiderable difference in the magnitude of activity. Considering with another fact that acylase activity is distributed in a variety of organisms, these findings offer an interesting suggestion to interpret the biological significance of acylase activity.

Of the acyl amino acids used as the substrate, acetylmethionine was most readily hydrolyzed by most plant acylase preparations and susceptibility was lowered in the order of chloroacetylphenylalanine, acetylglutamic acid and ε-benzoyl-α-acetyllysine. The last compound was insusceptible to plant acylases except in the case of mushroom under the conditions employed by the authors. In general, glycylmethionine was also as susceptible as acetylmethionine to most plant preparations.

Concerning the enzymes in mushroom, a number of reports have appeared and amidase in mushroom was employed for the resolution of racemic amino acid amides[9]. In respect of acylase activity in mushroom, however, not any report has been available. The experiments carried out with "Matsutake" (*Armilaria edoides*) revealed that in general, higher hydrolytic activities occur in mushroom, and that activity could hydrolyze ε-benzoyl-α-acetyllysine, which was insusceptible to all of the plant preparations tested. Also, in case of mushroom, peptidase activity towards glycylmethionine, was very high and chloroacetylphenylalanine was exceptionally more susceptible than acetylmethionine.

Acylase activities of various plants employing acetylmethionine as the substrate, are summarized in Table VII. As shown in the Table, in the case of enzymatic activities per mg of protein N, radish and mushroom showed the highest activity, pumpkin (*Cucurbita pepo*), cabbage and rice seedlings fol-

TABLE VII. ACYLASE ACTIVITY IN PLANTS

Plants \ Activity		μM/hr/N mg of protein	μM/hr/g of fresh matter
Vegetables			
Radish		21.21	0.08
Carrot		3.48	0.26
Cabbage		5.14	0.10
Spinach		2.74	0.18
Pumpkin	*pepo*	6.53	1.17
	moschata	3.64	0.30
Pulses			
Pea	Resting	1.12	2.72
	Immature	3.59	1.03
Soy-bean	Resting	0.26	1.14
	Seedling	0.57	2.51
Potatoes			
Potato		3.50	0.64
Sweet potato		0.43	0.09
Cereals			
Rice	Resting	3.17	0.74
	Seedling	4.64	1.44
Wheat	Resting	1.52	0.63
	Seedling	1.68	0.36
Barley	Resting	1.04	0.33
	Seedling	0.44	0.29
Fruits			
Mandarin orange	Peel portion	1.68	0.28
	Edible portion	0	0
Banana	Peel portion	0	0
	Edible portion	0	0
Fig		0.35	0.01
Mashroom			
"Matsutake"		10.42	4.48

---

[9] V. E. Price, J. P. Greenstein, L. Levintow and R.B. Kingsley, *Arch. Biochem.*, **26**, 92 (1950).

lowing. While, regarding the enzyme activities expressed in per unit g of fresh matter, mushroom was the highest and pulses, rice seedling, pumpkin (*Cucurbita pepo*) showed comparatively higher activities.

As a result of these experiments, a wide occurrence of acylase activity in plants was confirmed, although, activity was not so strong as in case of molds previously studied.

SUMMARY

(1) The occurrence of acylase activity in plants such as vegetables, pulses, cereals, potatoes, fruits and mushroom, has been confirmed for the first time.

(2) Of the acyl and glycyl amino acids tested, acetylmethionine and glycylmethionine were most readily hydrolyzed by most of the plant preparations tested, and susceptibility was lowered in the order of chloroacetylphenylalanine, acetylglutamic acid and ε-benzoyl-α-acetyllysine. The last mentioned compound was insusceptible to the plant acylase except in the case of mushroom.

(3) In the germination state of cereals and pulses, higher activities, although not so apparent, were often observed.

(4) Mushroom showed higher and somewhat different hydrolytic activities than the other higher plants tested.

Acknowledgements The authors are grateful to Prof. H. Mitsuda of Kyoto University and Dr. M. Fujisawa of this Laboratory for their helpful advice and encouragement in this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 376~380, 1959]

Studies on the Reduction of Terpenes with Sodium in Aqueous-ammonia

Part I. Synthesis of (−)-Menthol from (−)-Menthone

By Hiroo UEDA, Yasuo SHIBAHARA* and Sumio SHIMIZU**

Research Institute, Nagaoka & Co., Ltd, Motoyama, Kobe

Received December 8, 1958

On reduction of (−)-menthone by various methods, generally, a mixture of (−)-menthol and (+)-neomenthol has been obtained. In the present work, it is found that (−)-menthol can be prepared almost quantitatively from (−)-menthone by treatment with sodium in aqueous-ammonia.

Many attempts concerning the preparation of (−)-menthol from (−)-menthone by various reduction methods have previously been reported. However, the reduction products obtained by application of these methods was far from satisfactory from the economical point of view since previous methods gave a mixture of (−)-menthol and (+)-neomenthol without exception. It was an important theme in menthol

---

* Laboratory of Agricultural Chemistry, Faculty of Agriculture, University of Kyoto.

** Laboratory of Agricultural Chemistry, Faculty of Agriculture, Shinshu University.

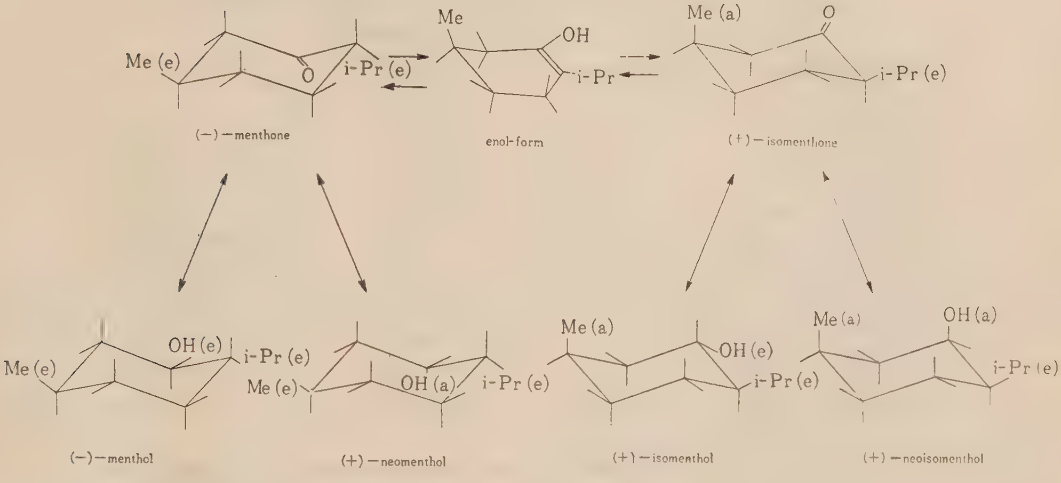


FIG. 1. Steric Configurations of the Isomeric Menthones and Menthols

industry to prepare (−)-menthol alone from (−)-menthone. In any syntheses of (−)-menthol, the primary problem was to attain a high yield of (−)-menthol simultaneously supressing formation of the remaining isomeric menthol.

Menthol contains three asymmetric carbon atoms. Applying concepts[1] of structures for cyclohexane derivatives, steric relationships of isomeric menthols and menthones are summarized in Fig. 1.

Upon oxidation, menthol and neomenthol give menthone, while isomenthol and neoisomenthol give isomenthone. Upon reduction of (−)-menthone by various methods, such as catalytic hydrogenation[2,3,4], aluminum-isopropoxide[5,6], lithium-aluminum-hydride[7] and sodium in organic solvents[8], (−)-menthol along with other isomers (mainly (+)-neomenthol) was prepared. Therefore, the various methods mentioned above are seemed to be unsatisfactory to obtain a high yield of (−)-menthol. Recently, Mori[9] obtained (−)-menthol from (−)-menthone in a yield of about 80% by treatment with sodium in benzene and water in which ammonium-chloride was dissolved. Also, Amagasa[10] reduced (−)-menthone by treatment with sodium in liquid-ammonia and obtained a (−)-menthol-rich product. The results reported by the workers mentioned above are summarized in Table I.

In the present investigation, it is found that aqueous-ammonia is also an useful solvent for the reduction of (−)-menthone by treatment with sodium. It gave more satisfactory results than the methods described above. This reaction proceeded more smoothly and completely, and the reduction products were obtained in an all-over yield of 90% and (−)-menthol content was about 90%. The experiments, as shown in Table II, were undertaken to determinate the required amounts of sodium and aqueous-ammonia, and the suitable temperatures for completion of reduction. When amounts of aqueous-ammonia were in an excess, the reaction proceeded uncompletely and unreacted menthone was remained. (Expt. 5-8)

---

[1] H. D. Orloff, *Chem. Revs.*, **54**, 368 (1954).
[2] G. Vavon and A. Couderg, *Compt. rend.*, **179**, 405 (1924).
[3] G. Vavon, *Compt. rend.*, **155**, 287 (1900).
[4] S. Hiraizumi, *Mem. Coll. Sci., Kyoto Imp. Univ.*, **1930**, 85.
[5] W. J. Grubb and J. Read, *J. Soc. Chem. Ind.*, **53**, 53T (1934).
[6] L. M. Jacmann and A. K. Macbeth, *J. Chem. Soc.*, **1949**, 2641.
[7] D. S. Noyce and D. B. Denney, *J. Am. Chem. Soc.*, **72**, 5743 (1950).
[8] H. Shinozaki and T. Nagasawa, *Repts. Imp. Ind. Research Inst., Osaka Japan*, **10**, No. 4, 85 (1929).
[9] K. Mori, Japan P. 2,239 (1951); *C. A.*, **47**, 4915.
[10] M. Amagasa, Japan P. 3,884 (1952); *C. A.*, **48**, 4592.

TABLE I. (−)-MENTHONE REDUCTION BY VARIOUS METHODS

Methods	Reduction Products $[\alpha]_D$	 (−)-Menthol content %	Byproduct
Catalytic hydrogenation			
Pd	−4° ~ −10°		(+)-neomenthol (2)
Pt	−10.6°		(3)
Ni 260°, 50 atm.	−14.86°		(+)-neomenthol (4)
Aluminum-isopropoxide	−0.9°	30	(+)-neomenthol (5,6)
Lithium-aluminum-hydride	−28.00°	71	(+)-neomenthol (7)
Metallic sodium			
in ethanol	−23.0°		(8)
in benzene, water and ammonium-chloride		80	(9)
in liquid-ammonia	−34°		(10)

TABLE II. REDUCTION OF (−)-MENTHONE (10.0 g) WITH SODIUM IN AQUEOUS-AMMONIA

Expt. No.	Reagents Aqueous ammonia ml	 Benzene ml	 Sodium g	Reaction Temp. °C	Reduction Products Yield %	 M.p. °C	 $[\alpha]_D^{20}$	 Unreacted menthone %
1	20+20	20+10	7.4	3– 6	92	37–39	−43.8	0.6
2	20+20	20+10	6.4	3– 6	97	34–37.5	−41.6	2.2
3	20+20	20+10	5.4	3– 6	95	34–37	−42.3	0.1
4	20+10	20	4.5	3– 6	94	23–31.5	−39.6	13.9
5	50	20	7.1	3– 6	97	liq.	−35.1	34.8
6	40	20	5.7	3– 6	97	18–25.5	−37.8	20.4
7	30	20	4.2	3– 6	95	liq.	−34.8	34.2
8	20	20	2.8	3– 6	95	liq.	−33.3	46.1
9	30+20	10	9.0	15–25	95	34–38	−41.2	0.0
10	30	10	8.0	15–25	90	23–32.5	−32.8	16.1
11	30	10	7.0	15–25	90	liq.	−28.8	29.9
12	30	10	6.0	15–25	93	19–32.5	−31.7	21.2
13	30	10	5.0	15–25	93	liq.	−29.7	38.0

When the reaction temperatures were too high, in the range 15-25°, the reactions proceeded uncompletely and the contents of isomeric menthol in products increased. (Expt. 9-13)

The best result was obtained in the condition as follows. (Expt. 3) In a stirred mixture of 10 g (−)-menthone in 20 ml aqueous-ammonia and 20 ml benzene, maintained at 3° to 6° in an ice-salt bath, 5.4 g of wiry sodium reduced (−)-menthone to (−)-menthol completely. The product was worked up in the usual manner and after distillation a solid crude (−)-menthol was obtained in a yield of about 95%. Asuming that the byproducts are (+)-neomenthol alone, the proportion of (−)-menthol and (+)-neomenthol in the products is indicated in Table III.

The prodominant production of (−)-menthol might conclude that (−)-menthone is not inverted to (+)-isomenthone during this reduction.

Using a water instead of aqueous-ammonia, it was difficult to control the reaction temperature, and the reaction proceeded uncompletely. The reduction product was shown to be of lower melting point and lower optical rotation in comparison with those of the above-described

TABLE III. THE PROPORTIONS OF (−)-MENTHOL AND (+)-NEOMENTHOL IN REDUCTION PRODUCTS OF (−)-MENTHONE

Expt. No.	Unreacted menthone %	(−)-Menthol %	(+)-Neomenthol %	$\frac{(-)\text{-Menthol}}{\text{Total-Menthol}}$ %
1	0.6	91.5	7.9	92.0
2	2.2	87.2	10.6	89.1
3	0.1	89.7	10.2	90.5
4	13.9	76.0	10.1	88.2

method. This will be due to a higher content of isomeric menthols. Consequently, it is clearly shown that aqueous-ammonia is indispensable for this reduction, and the reduction mechanism in this case will be explained according to Greenlee[11].

EXPERIMENTAL

(−)-Menthone (−)-Menthone was prepared by oxidation of (−)-menthol with Beckmann's[12] chromic acid mixture, b.p. 91.2–92.0°/14.5 mm, $[\alpha]_D^{22}$–28.3° (homog.), n_D^{23} 1.4476, Yield 95.4%.

Reduction of (−)-menthone (Table II. Expt. 3) To a stirring mixture of 10.0 g (−)-menthone in 20 ml benzene and 20 ml aqueous-ammonia (d 0.904), 5.4 g of wiry sodium was added portionally. During the addition, reaction temperature was maintained from 3° to 6° in an ice-salt bath. As sodium-hydroxide was crystallizing, 20 ml aqueous-ammonia was added when necessary. After sodium had disappeared, allowed to stirring an additional thirty minutes, the organic phase was extracted with ether and the ether extract was washed with water and diluted hydrochloric acid solution and finally dried over sodium-sulfate. After removal of the ether, the residue was distilled to gave 9.5 g, b.p. 104–105°/16.5 mm. This fraction crystallized on standing, m.p. 34–37°, $[\alpha]_D^{20}$–42.3° (in methanol). The unreacted menthone content was 0.1%. One recrystallization from nitromethane brought the melting point up to 41.5–42.0°, $[\alpha]_D^{20}$–48.5° (in methanol). Its 3,5-dinitrobenzoate was shown as m.p. 153.5–154°, not depressed by admixture with (−)-menthyl-3,5-dinitrobenzoate.

Determination of unreacted menthone A modification of Sabetay's[13] method was used in the determination of unreacted menthone. To a 1.0 g reduction product and 1.0 g calciumcarbonate, 25 ml of 5%-hydroxylamine-hydrochloride alcoholic solution was added. After being allowed to stand over night, 25 ml of 1 N-hydrochloric acid solution was added to it under cooling and was titrated with a N/2-potassium-hydroxide alcoholic solution. As the indicator, 0.1 ml of 0.2%-alcoholic solution of mixture of dimethylyellow and methylenblue was used, instead of bromphenolblue. This indicator gave a better result than bromphenolblue for the change of color at the end-point.

Isolation of unreacted menthone and isomeric menthol Reduction products (Table II., Expt. 1–3), 22.3 g, was recrystallized from nitromethane to give 19.9 g (−)-menthol. From this mother liquid, an oily fraction 474 mg, b.p. 101–102°/18 mm was obtained. This fraction gave 519 mg *p*-nitrobenzoate, but no crystalline could be isolated. Therefore, this *p*-nitrobenzoate was hydrolysed with 10 ml of N/2-potassium-hydroxide alcoholic solution, and crude 3,5-dinitrobenzoate 622 mg, m.p. 120–130° was obtained. After crystallization twice from methanol, it gave needles crystal 270 mg, m.p. 154–155°, undepressed by admixture with (−)-menthyl-3,5-dinitrobenzoate, and depressed to 129.5–132° by admixture with (+)-neomenthyl-3,5-dinitrobenzoate.

An attempt to separate the (+)-neomenthyl-3,5-dinitrobenzoate from this mother liquid was unsuccessful, but the unreacted menthone was isolated as crude semicarbazone, m.p. 172–176° (151 mg) from the steam-distillate of crude *p*-nitrobenzoate. One recrystallization yielded 100 mg of semicarbazone, m.p. 187–188°, undepressed by admixture with (−)-menthone-semicarbazone.

The proportion of (−)-menthol and (+)-neomenthol (Table III) From the optical rotation of the reduction products and known values[14] $[\alpha]_D$–29.6°, −49.4° and +19.7° for (−)-menthone, (−)-menthol and (+)-neomenthol respectively, the proportions of (−)-menthol and (+)-neomenthol were calculated.

Reduction of (−)-menthone using water instead of

---

[11] K. W. Greenlee and W. C. Fernellius, *J. Am. Chem. Soc.*, **64**, 2505 (1942).

[12] *Organic Synthesis*, Vol. I, p. 340.

[13] Sabetay, *Bull. Soc. Chim. France*, (5), **5**, 1419 (1938).

[14] J. L. Simonsen, "The Terpenes", Cambridge Univ. Press, 1953, Vol. I, p. 241, 243.

aqueous-ammonia To a stirring mixture of 10.0 g (−)-menthone in 20 ml water and 20 ml benzene, 6.7 g of sodium was added portionally, reaction temperature was fixed at 3° to 19° during the reaction. The reduction product was worked up in the usual manner, and a solid product was obtained. Yield 8.8 g. From this product (8.0 g) unreacted menthone (6.1%) was removed by formation of semicarbazone, m.p. 184–185° (0.5 g). The product (menthone free) was found to be m.p. 25–32° and $[\alpha]^{20}_{D}$−37.8° (in methanol) ((−)-menthol content 82.6%).

Acknowlegement The authors are indebted to Prof. Dr. S. Takei, Prof. Dr. T. Mitsui and Dr. M. Nakajima in the University of Kyoto for their cordinal advice and helpful suggestions.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 380~383, 1959]

Studies on the Reduction of Terpenes with Sodium in Aqueous-ammonia

Part II. Synthesis of (+)-Isomenthol from Inverted Menthone

By Hiroo UEDA and Sumio SHIMIZU*

Research Institute, Nagaoka & Co., Ltd. Motoyama, Kobe

Received December 8, 1958

The synthesis of (+)-isomenthol has been studied by several groups of workers, but their experiments proved to be far from satisfactory. In this investigation, it is now shown that (+)-isomenthol is obtained in a better yield from inverted menthone, a mixture of (−)-menthone and (+)-isomenthone, by a new reduction method using sodium in aqueous-ammonia.

(+)-Isomenthol has been prepared by the reduction of (−)-piperitone[1] or by diazo-reaction[2] of (+)-isomenthylamine which be obtained from inverted menthone via menthoneoxime. However, it was difficult to obtain isomenthol in a pure state, since (−)-piperitone was easily isomerized to (±)-piperitone by alkali, and the diazo method was very tedious and the yield was much lower. Both methods were found to be far from satisfactory.

In the first paper of this series, it has been shown that (−)-menthone was reduced to (−)-menthol almost quantitatively by treatment with sodium in aqueous-ammonia, and (−)-menthone was not inverted to (+)-isomenthone in this reaction.

In this investigation, it is found that pure (+)-isomenthol can be prepared from inverted menthone[3], namely a mixture of (−)-menthone and (+)-isomenthone, by the reduction

---

* Laboratory of Agricultural Chemistry, Faculty of Agriculture, Shinshu University.

[1] J. Read, *J. Chem. Soc.*, **1927**, 1276.

[2] H. Ueda and T. Mitsui, *J. Agr. Chem. Soc. Japan*, **28**, 945 (1954).

[3] E. Beckmann, *Ann.*, **250**, 325 (1889).

TABLE I. FRACTIONAL DISTILLATION OF INVERTED MENTHONE

Fract. No.	b.p. /4–5 mm. °C	Yield g	n_D^{20}	d_{18}^{18}	$[\alpha]_D^{18}$	(+)-Isomenthone content %
1	68.0–68.7	25.9	1.4509	0.8967	+ 3.3	26.4
2	68.7–69.8	24.1	1.4510	0.8967	+ 3.3	26.4
3	70.0–70.2	25.1	1.4514	0.8978	+ 7.8	30.0
4	70.7–71.0	24.6	1.4513	0.8976	+13.4	34.5
5	68.8–69.8	23.9	1.4516	0.8990	+20.2	40.0
6	69.8–70.0	25.3	1.4521	0.8990	+36.5	53.0
7	70.0–70.2	20.0	1.4527	0.9005	+58.0	70.3
8	70.2–70.3	9.4	1.4535	0.9024	+76.5	85.1
9	70.3–71.2	4.6	1.4601	0.9048	+69.2	79.3
10	Residue	1.2	—	—	—	—

TABLE II. REDUCTION OF INVERTED MENTHONE WITH SODIUM IN AQUEOUS-AMMONIA

Expt. No.	Inverted menthone		Reagents			Reduction Product		
	g	(+)-Iso-menthone %	Aqueous-ammonia ml	Benzene ml	Sodium g	m.p. °C	Unreacted menthone %	Yield %
1	10.0	49.8	20	20+20	6.6	32–38	1.5	90
2	10.0	53.0	20+20	20+20	6.4	34–45.5	2.9	89
3	14.9	53.0	40+20	40	9.9	40–42.2	2.8	93
4	10.0	68.8	20+20	20	6.4	42–50.5	5.7	91
5	15.6	69.7	40+32	40	11.6	40–46	15.2	92
6	10.0	70.3	20+20	20+20	6.4	42–51	4.7	91
7	11.5	81.3	20+20	20	6.6	49.5–54	8.5	90

described above.

Inverted menthone was obtained from (−)-menthone by treatment with sulphuric acid, and seemed to be a mixture of (−)-menthone, 55.5% and (+)-isomenthone, 44.5%. Then fractional distillation was caaried out to give 10 approximately equal fraccions (Table I). On this distillation, about 50% of total (+)-isomenthone was condensed to fractions 6-9, which were summed up to make about one-third of original inverted menthone. When oil-bath temperature was kept in the range of 100-105° during this distillation, it was found that (+)-isomenthone is not changed back into (−)-menthone.

Each fraction was reduced by treatment with sodium in aqueous-ammonia according to the method described in our previous paper. These experimental results are shown in Table II. Unreacted menthone, about 1-15%, was removed by formation of semicarbazone. The resulted menthol mixtures (menthone free) showed the following physical characters as given in Table III. (+)-Isomenthol contents in this table were estimated by an optical rotation method, assuming that these menthol mixtures are consisted of (−)-menthol and (+)-isomenthol only.

TABLE III. REMOVAL OF UNREACTED MENTHONE

Expt. No.	Reduction Menthol	Menthol Mixture (menthone free)			
	g	m.p. C°	$[\alpha]_D^{20}$	(+)-Isomenthol content %	Yield %
1	8.4	35–37	−18.0	41.1	89
2	8.3	39–42	−17.6	41.6	91
3	13.4	32–38	−15.7	44.1	95
4	8.6	40–51	−11.0	50.3	80
5	13.8	43–53.5	−8.0	54.2	91
6	8.6	47–54.5	−4.7	58.5	98
7	9.9	50–58.5	−0.7	63.7	100

Strictly speaking, small amounts of (+)-neomenthol and (+)-neoisomenthol are possible to exist in these menthol mixtures. Percentage of each (+)-isomenthol content was lower than theoretical values which was expected from (+)-isomenthone contents in original inverted menthone. It is shown that (+)-isomenthone is considerably changed back into (−)-menthone in this reduction, although (−)-menthone was not inverted to (+)-isomenthone, as described in our previous paper.

If these facts are to be accepted, the rate of inversion of (+)-isomenthone is considerably more rapid than that of (−)-menthone in alkali medium. These differences between (+)-isomenthone and (−)-menthone will be expected, since Read[4] has reported that (±)-menthol formation was predominant over (±)-isomenthol when (±)-isomenthone was reduced by sodium in ethanol.

By recrystallization from acetone or *n*-hexane, (+)-isomenthol, m.p. 70-75°, was obtained as shown in Table IV. One recrystallization from *n*-hexane gave pure (+)-isomenthol, m.p. 82-83°, $[\alpha]_D^{20}+26.6°$.

TABLE IV. RECRYSTALLIZATION OF MENTHOL MIXTURE (menthone free)

Expt. No.	Menthol Mixture g	(+)-Isomenthol Yield g	m.p. °C	Yield for Theor. %
1–2	11.6	1.3	70–75	27.1
3	10.8	2.0	70–75	42.0
4	4.9	1.4	69–74	56.9
5	8.8	3.6	73.5–76.5	75.5
6	6.4	3.5	68–73	93.6
7	7.5	4.0	75–79	83.7

EXPERIMENTAL

Inverted menthone (−)-Menthone was inverted according to the procedure of Beckmann. To 400 ml of freezed concentrated sulphuric acid, 106 g of (−)-menthone ($[\alpha]_D^{18}-27.2°$) was added under stirring over thirty minutes, and was allowed to stand one night in an ice-box. This reaction mixture was warmed at 30° in a water-bath under stirring for 1.5 hrs, and was then poured onto ice-water. The ether extract was washed with water, and finally dried over sodium sulfate. After removal of ether, the residue was distilled to give inverted menthone, 100 g, b.p. 83–85°/11 mm, $[\alpha]_D^{14}+27.6°$ (homog.), yield, 94.5%. The (+)-isomenthone content was determinated to be 45.9% by an optical rotation method.

Fractional distillation of inverted menthone (Table I) The inverted menthone, 188 g, ($[\alpha]_D^{12}+25.8°$, (+)-isomenthone 44.5%) was fractional distilled in a 17×1250 mm Stedman Column packed with nickel-chrom-rings (0.4×4 mm) (approx. 50–60 theor. plates, reflux-ratios 10 : 1) to give 10 approximately equal fractions as shown in Table I. The (+)-isomenthone contents were estimated by the optical rotation method. The mixture of fractions 1–4, 98 g, which was found to contain poor (+)-isomenthone (29.3%), was inverted again to give inverted menthone, 93 g ($[\alpha]_D^{18}+23.1°$, (+)-isomenthone 42.3%) yield, 94.9%.

Reduction of inverted menthone (Table II) Each fraction obtained by distillation was reduced according to the procedure described in Part I. For example (Expt. 6), to a stirring mixture of 10.0 g inverted menthone (Fract. 7, (+)-isomenthone 70.3%) in 20 ml of benzene and 20 ml of aqueous-ammonia (d 0.904), 6.4 g of wiry sodium was added portionally. During this addition, the reaction temperature was maintained at 3° to 6° in an ice-salt bath. As the reaction proceeded, additional 20 ml of aqueous-ammonia and 20 ml of benzene were added. The reduction product was 9.1 g of a solid menthol mixture, m.p. 42–51°, which was contaminated with 4.7% of unreacted menthone.

Removal of unreacted menthone (Table III) The remained unreacted menthone (1.5–15.2%) was removed as semicarbazone. For example (Expt. 6), to a solution of semicarbazide-hydrochloride 1 g, sodium-acetate-anhydride 0.8 g and water 2 ml, the menthol mixture, 8.6 g, (unreacted menthone 4.7%) was added. To this mixture methanol was added dropwise to be clear solution. After one night, methanol was removed under diminished pressure and the residue was extracted with ether. The ether extract was washed with water and 5%-potassium-hydroxide solution and finally dried over sodium sulfate. After removal of ether, the residue was distilled to give 8.0 g menthol mixture (menthone free), m.p. 47–54.5°, $[\alpha]_D^{20}-4.7°$ (in methanol), in 97.6% for theoretical yield, while menthone-semicarbazone was remained in the distillation residue.

(+)-Isomenthol (Table IV) (+)-Isomenthol was obtained in a pure state from each menthol mixture

---

[4] J. Read, *J. Chem. Soc.*, **1925**, 2782.

(menthone free) by recrystallization from acetone or *n*-hexane. The yields from the (+)-isomenthol-rich fractions were found satisfactory by once or twice recrystallizations (Expt. 5–7). On the (+)-isomenthol-poor fractions several recrystallizations were required but the yields were unsatisfactory (Expt. 1–3). For the synthesis of (+)-isomenthol, therefore, it is an important point to make an inverted menthone fraction containing (+)-isomenthone in a rich amount by fractional distillation or by some other method. Each of the crude (+)-isomenthol (Table IV) was put together. This total crystal, 18.5 g, was recrystallized from *n*-hexane to give pure (+)-isomenthol, 14.6 g, $[\alpha]_D^{20}+26.6°$ (in methanol), m.p. 82–83°, undepressed on admixture with a sample prepared by diazo-reaction of (+)-isomenthylamine. Its 3,5-dinitrobenzoate melted at 146–147°, and was not depressed by mixture with (+)-isomenthyl-3,5-dinitrobenzoate, m.p. 146.

Acknowlegement The authors are indebted to Prof. S. Takei, Prof. T. Mitsui, Prof. M. Nakajima and Assoc. Prof. T. Hashizume in the University of Kyoto for their kind advice and suggestion. The authors also wish to express his sincere thanks to Mr. K. Fujimoto for assistance in carrying out the experiment.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 383~388, 1959]

Effects of Phosphatase and Phosphoglucomutase on the Determination of Phosphorylase Activities in Crude Extracts from Plant Materials

By Michinori NAKAMURA

Department of Agricultural Chemistry, the University of Tokyo

Received December 16, 1958

The extent to which phosphatases interfere with the determination of phosphorylase activity in crude extracts from plant materials by hydrolyzing glucose-1-phosphate was determined. Sodium fluoride was an effective, although not satisfactory, inhibitor of phosphatase. Evidence was presented to show that in crude extract of broad bean seeds glucose-1-phosphate was hydrolyzed most probably via glucose-6-phosphate.

Phosphorylase transfers reversibly the glucosyl group from G–1–P* to the non-reducing end of the pre-existing amylose chain by the mechanism which can be formulated as follows:

$$(C_6H_{10}O_5)_n + C_6H_{11}O_5 \cdot OPO_3^= \rightleftarrows (C_6H_{10}O_5)_{n+1} + HOPO_3^=$$

Phosphorylase activity can therefore be determined by various ways. Among them, the method most frequently used is the one in which the initial rate of the liberation of P*i* is taken as a measure of its activity. However, many factors interfere with the determinanion of phosphorylase activities in crude extracts of plant materials. The effects of amylases on starches used as primer or formed as reaction products have already been reported[1,2,3].

---

* The following abbreviations are used throughout this paper; G-1-P, α-D-glucose-1-phosphate; G-6-P glucose-6-phosphate; P*i*, inorganic orthophosphate.

1) H. K. Porter, *Biochem. J.*, **47**, 476 (1950).

2) M. Nakamura, *Nippon Nôgei-kagaku Kaishi* (J. Agr. Chem. Soc. Japan), **24**, 302 (1951).

3) H. K. Porter, *J. Exptl. Botany*, **4**, 44 (1953).

Inasmuch as G-1-P is the substrate not only of phosphorylase but also of many other enzymes, it is conceivable that there may exist many ways of transformation of G-1-P into other phosphorus compounds. The effects of these reactions on the determination of phosphorylase activity may be, however, summed up in two points. First, the effect of the decrease in the concentration of G-1-P due to its conversion into other phosphorus compounds. This effect may be circumvented by adding a high concentration of G-1-P in the enzymic reaction mixture, thus a sufficient level of G-1-P being maintained during the course of the determination. However, in cases where decreases in G-1-P are very rapid, it may be difficult to obtain the initial rate of the phosphorylase reaction. Also, when phosphorylase activity is determined by the formation of G-1-P in the phosphorolysis of starch, for example, by the procedure of Takasugi et al[4], conversion of G-1-P formed as the reaction product into other acid-resistant phosphorus compounds may cause large errors.

Second, the effect of hydrolysis of G-1-P to form inorganic phosphate by the action of phosphatase. It is evident that any hydrolysis of G-1-P will give an apparently increased phosphorylase activity. It is not known as yet to what extent phosphatases would actually affect the determination of phosphorylase activities, and this point is the main concern of this paper. Effects of enzymes converting G-1-P into acid-resistant phosphorus compounds are also included.

EXPERIMENTAL

Materials.

G-1-P: Crystalline dipotassium salt of G-1-P was prepared and purified by the method of Hassid and McCready[5] with some modifications[6].

G-6-P: Barium salt of G-6-P was prepared by the enzymic method of Inoue et al.[7]

Glycerophosphate: Calcium glycerophosphate, Merck, was used without further purification.

β-Amylase from Sweet Potatoes: Twice recrystallized β-amylase, which was prepared by the method of Balls et al.[8], was generously supplied by Prof. M. Itô. Assay of β-amylase activity was carried out as reported previously[9].

Preparation of Crude Extracts from Plant Materials: Plant materials were homogenized in the cold with two volumes of distilled water, and the homogenates were centrifuged at 3,000 r.p.m. for 5 min. The supernatant fluids were stored in an ice-box until use.

Procedures.

Phosphorylase Activity: Activity measurement was carried out as reported previously[9].

Chemical Analysis of Phosphorus Compounds: P*i* was determined by the method of Nakamura[10]. G-1-P was determined by the difference of P*i* before and after hydrolysis in 1 N sulfuric acid for 7 min. in a boiling water bath. The value thus obtained may represent the acid-labile P of -G1-P, since conceivably there may not exist any other acid-labile P compounds in the phosphorylase reaction mixture. Changes in acid-resistant P during the course of enzymic reaction were determined by the difference of P*i* after hydrolysis in 1 N sulfuric acid for 7 min. in a boiling water bath. Alternatively, acid-resistant P was determined as follows: An aliquot of the reaction mixture was made 1 N with respect to sulfuric acid, and the mixture was heated for 7 min. in a boiling water bath. After cooling, the mixture was treated with 3 volumes of magnesia mixture[11] to remove P*i*. Aliquot of the neutralized filtrate was used for the determination of P*i* and total P. The difference of these two values represents acid-resistant P.

Detection of Starch: One-tenth ml. of the sample solution was mixed with 6-8 ml. of water containing 0.2 ml. of 0.2% I_2-2% KI solution and the resulting mixture was made up to 10 ml. with water. The optical density at 610 mμ of the solution was measured

---

[4] N. Takasugi and E. Itô, *Symp. on Enzyme Chem.*, **6**, 50 (1950).

[5] W. Z. Hassid and R. M. McCready, in *Biochemical Preparations*, Vol. 4, p. 63. John Wiley & Sons (New York, 1955).

[6] K. Mori and M. Nakamura, This Bulletin, **23**, 272 (1959).

[7] Y. Inoue, K. Onodera and T. Itô, *Nippon Nôgei-kagaku Kaishi*, **30**, 59 (1956).

[8] A. K. Balls, M. K. Walden and R. R. Thompson, *J. Biol. Chem.*, **173**, 9 (1948).

[9] M. Nakamura, K. Yamazaki and B. Maruo, *Nippon Nôgei-kagaku Kaishi*, **24**, 197 (1951).

[10] M. Nakamura, *Nippon Nôgei-kagaku Kaishi*, **24**, 1 (1950).

[11] G. A. LePage, in W. W. Umbreit, R. H. Burris, and J. F. Stauffer, *Manometric Techniques*, p. 276. Burgess Publ. Co. (Minneapolis, 1957).

TABLE I. PHOSPHATASE ACTIVITIES OF CRUDE EXTRACTS FROM SOME PLANT MATERIALS

Each reaction mixture contains, in a total volume of 5 ml., 1 ml of 0.25 M maleate buffer, pH 6.0, 1 ml of 0.1 M calcium glycerophosphate or sodium G-6-P, 1 ml of the crude extract, and sodium fluoride in concentrations as indicated in the table. Temperature: 37±0.1°C. One unit of enzymic activity is defined as the amount of enzyme which liberates 0.1 mg of *Pi* in 10 min. under the conditions described above. Figures in the table are expressed in terms of phosphatase units per ml of the crude extract. Figures in parentheses represent remaining activities as compared to the original solutions without added fluoride.

Plant materials	Glycerophosphate			G-6-P		
	No NaF	0.01 M NaF	0.02 M NaF	No NaF	0.01 M NaF	0.02 M NaF
Potato tubers	0.576	0.308 (54%)	0.250 (43%)	0.403	0.058 (14%)	0.039 (10%)
Rice leaves	0.768	0.557 (72%)	0.557 (72%)	0.632	0.134 (21%)	0.058 (9%)
Pumpkin leaves	1.66	0.905 (55%)	0.923 (56%)	1.12	0.173 (15%)	0.116 (10%)
Broad bean seeds	0.423	0.346 (82%)	0.211 (50%)	0.326	0.154 (47%)	0.072 (22%)
Mung bean seedlings (2-day old)	2.02	0.786 (39%)	0.710 (35%)	1.17	0.269 (23%)	0.154 (13%)

by the Hirama photoelectric colorimeter by using a 1 cm. cuvette.

Determination of Reducing Sugars: Aliquot of the phosphorylase reaction mixture was deproteinized according to the method of Somogyi[12)], and the amount of reducing sugar in the deproteinized filtrate was determined by the colorimetric method of the same author[13)].

Paper Chromatography of Sugars: Aliquot of the deproteinized filtrate obtained above was concentrated to dryness in a vacuum desiccator over sulfuric acid. The residual matter was dissolved in a small volume of water and subjected to paper chromatographic separation by using BuOH-EtOH-H_2O (10 : 1 : 2) or BuOH-pyridine-H_2O (6 : 4 : 3) as the solvent. The filter paper used was Tôyô No. 50. Multiple ascending method was used. Sugars were detected by the benzidine-trichloroacetic acid reagent of Bacon and Edelman[14)]. Authentic samples of glucose and maltose were used as the reference standards.

RESULTS

Phosphatase Activity of Crude Plant Extracts and Its Inhibition by Sodium Fluoride.

In crude extracts of potatoes and sweet potatoes in which both phosphorylase and phosphatase activities are rather high, only very slight liberation of *Pi* is observed, when due precaution is paid to eliminate any primer contaminants from the reaction mixtures[15),16)]. This fact may indicate that the phosphatase activity which hydrolyzes G-1-P is practically negligible, if not at all, in these plant extracts.

Since there has not been any report indicating the presence of a phosphatase which hydrolyzes G-1-P directly, the most probable pathway to liberate *Pi* from G-1-P may be the one which works via G-6-P by the cooperative action of phosphoglucomutase and phosphatase. Although it is known that phosphoglucomutase is widely distributed in plants, the presence of a phosphatase which hydrolyzes G-6-P is known only in a small number of plant materials. Therefore, activities of crude extracts from some plant materials to hydrolyze G-6-P and the inhibitory effets of sodium fluoride on them were determined under the conditions for the determination of phosphorylase activity. Phosphatase activities of these extracts on glycerophosphate were also determined for comparison. The results are given in Table I.

As can be seen from the table, phosphatase activity hydrolyzing glycerophosphate was rather resistant to fluoride and increasing concentrations of fluoride had not marked effect on the inhibition of phosphatase. On the other hand, phosphatase hydrolyzing G-6-P was fairly sensitive to fluoride, most of them being inhibited more than 50% by 0.01 M and more than 80% by 0.02 M of sodium fluoride. It is interesting to note that, although phosphatase activities of

---

12) M. Somogyi, *J. Biol. Chem.*, **160**, 69 (1945).
13) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).
14) J.S.D. Bacon and J. Edelman, *Biochem. J.*, **41**, 711 (1956).
15) M. Nakamura, This Bulletin, in press.
16) M. Nakamura, *Nippon Nôgei-kagaku Kaishi*, **25**, 413 (1952).

these extracts towards G–6–P and glycerophosphate were fairly parallel, their behavior towards fluoride showed a quite different picture. Thus, these two phosphatase activities may represent isodynamic enzymes.

Effect of Phosphatase and Its Inhibition by Sodium Fluoride in the Determination of Phosphorylase Activity.

The extent to which phosphatase contaminants interfere with the determination of phosphorylase activity can be measured in two ways. First, phosphatase activity may be inhibited without any effect on phosphorylase activity. Second, phosphorylase activity may be inhibited specifically without concomitant inhibition of phosphatase activity. For the former approach, sodium fluoride was used. For the latter approach, a large excess of β-amylase was added to the phosphorylase reaction mixture without added primer. Experiments pertaining to these approaches by using crude extracts of potatoes and pumpkin leaves as the enzyme source are shown in Fig. 1, A and B, respectively.

When most of the phosphatase activity is assumed to be inhibited by fluoride, the difference between curves I and II may represent the effect of phosphatase contaminants. Since, however, the inhibition of phosphatase activity by fluoride may not be complete, the difference may represent the minimum fraction of phosphatase activity. On the other hand, the difference between curves IV and V may show the true phosphatase activity, because most of the phosphorylase activity might have been inhibited nearly completely in the presence of a large excess of β-amylase. It is interesting to note that the difference between curves IV and V is greater than that between curves I and II. This fact may be interpreted, as Porter[3] has already pointed out, that, when phosphorylase is active, phosphorylase competes with phosphatase for G–1–P and inhibits phosphatase activity. Activity shown in curve III was dependent on the amount of primer contained as contaminant in G–1–P and/or in the enzyme solution. Therefore, curve III varied greatly from sample to sample.

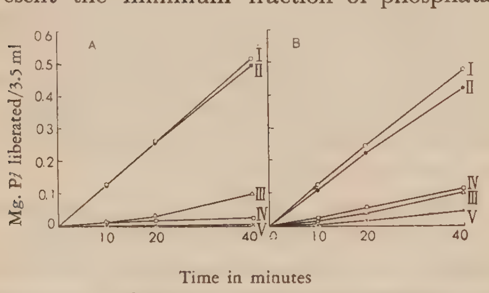


FIG. 1. Effects of Phosphatase Contaminants on the Determination of Phosphorylase Activities.

Each reaction mixture contains, in a total volume of 3.5 ml., 1 ml. of 0.25 M maleate buffer, pH 6.0, 0.5 ml. of 0.2 M G-1-P, and 0.3 ml. of potato extract (A) or 1 ml. of pumpkin leaf extract (B). Further additions to each curve are: I, 0.5 ml. of 5% soluble starch; II, 0.5 ml. of 5% soluble starch and 0.5 ml. of 0.1 M NaF; III, 0.5 ml. of 0.1 M NaF; IV, 0.5 ml. of β-amylase solution (412 units/ml.); and V, 0.5 ml. each of β-amylase solution and 0.1 M NaF. Temperatute: 37±0.1°C.

As can be seen from these figures, the curves were qualitatively very similar. Quantitatively, however, contribution by phosphatase contaminants varied with the source of enzymes. Thus, interference by phosphatase was negligibly small in potato extracts, while approximately 10% of the liberation of P*i* was due to phosphatase activity in the pumpkin leaf extract. In crude extracts from leaves of spinach, turnip (*Brassica rapa*), and rape (*B. cernua*), broad bean seeds, and 2-day old mung bean seedlings, the contribution by phosphatase was approximately 5–10%. In crude extracts from leaves of rice and barley, the contribution often reached 20%.

In curve V of Fig. 1, B, there was a small, but definite, increase in P*i* during the course of the reaction. This showed that the inhibition of phosphatase and/or phosphorylase by fluoride and/or β-amylase, respectively, were not complete, because under the conditions of these experiments, hydrolysis of G–1–P by potato extract was negligibly small, as can be seen in a similar curve of Fig. 1, A.

Conversion of G–1–P by Enzymes Other Than Phosphorylase.

Curves IV of Fig. 1 represent liberation of P*i* from G–1–P in the virtual absence of phosphorylase activity. Thus, these curves may be due mainly to phosphatase activity acting directly or indirectly on G–1–P. It is expected, therefore, that analyses of phosphorus compounds and reducing sugars under these conditions may reveal the nature of the phosphatase action on G–1–P.

When G–1–P was assumed to be hydrolyzed after it had once been converted into other phosphorus compounds (most probably G–6–P), accumulation of some acid-resistant P compounds would be expected. And this might be dependent on the relative ratio of phosphoglucomutase, for instance, and phosphatase activities. In fact, only negligible amount of acid-resistant P was accumulated in the reaction mixture when crude extract of potatoes or mung bean seedlings was used as the enzyme. Since the activity of phosphoglucomutase was very low in potato extract, it was natural that it was difficult to detect acid-resistant P in the enzymic digest. Results obtained with crude extract of broad beans, in which phosphoglucomutase activity is very high, are shown in Fig. 2. Similar experiments, in which 0.01 M of magnesium chloride or 0.01 M of sodium fluoride is added to activate phosphoglucomutase or to inhibit phosphatase activity, respectively, are also included in the figure.

It can be seen from Fig. 2, B, that magnesium ions had a powerful activating effect on phosphoglucomutase. Thus, conversion of G–1–P into acid-resistant P was almost complete in 1.5 hr. in the presence of added magnesium ions. Unexpectedly, 0.01 M fluoride had no inhibitory effect on phosphoglucomutase activity, because as was evident from the comparison of Fig. 2, A and C, the sum of P*i* and remaining acid-resistant P or the amount of remaining G–1–P at any time was almost the same for these two reaction mixtures.

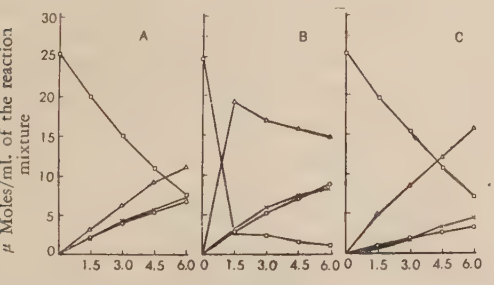


FIG. 2. Conversion of G-1-P in Crude Extract of Broad Bean Seeds in the Presence of β-Amylase.

Each reaction mixture contains, in a final volume of 7 ml., 2 ml. of 0.25 M maleate buffer, pH 6.0, 2 ml. of broad bean extract, 1 ml. of sweet potato β-amylase (412 units/ml.), and 1 ml. of 0.2 M G-1-P. B and C contain, in addition to the above, 1 ml. each of 0.1 M $MgCl_2$ or 0.1M NaF, respectively. Temperature; 37±0.1°C. Symbols used are: open circles, P*i*; crosses, reducing sugars (calculated as glucose); triangles, acid-resistant P; and squares, acid-labile P.

The amount of reducing sugars, calculated as glucose, was almost the same as the amount of P*i*. This showed that liberation of reducing sugar was due to hydrolysis of G–1–P and not to the combined action of phosphorylase and β-amylase activities to form maltose. This was supported by the finding that paper chromatography of the reaction mixtures revealed the presence of a large amount of glucose and only a small amount of maltose. In all these experiments, starch formation was not observed by the iodine color reaction.

Conversion of G–1–P Formed in the Phosphorolysis of Starch.

When phosphorylase activity is assayed by determining the amount of G–1–P formed in the phosphorolysis of starch, it is expected that any contaminating phosphoglucomutase will cause a marked influence on the results obtained. However, as can be seen in Fig. 3, at least in the early stage of the reaction, the presence

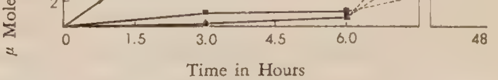


FIG. 3. Phosphorolysis of Starch by Crude Extract of Broad Bean Seeds.

One ml. of the reaction mixture contains 250 μmoles of P*i*, 10 mg. of soluble starch, and 0.2 ml. of broad bean extract. Other additions are: triangles, 10 μmoles of $MgCl_2$; squares, 10 μmoles of NaF; circles, no addition. Open signs, acid-labile P; closed signs, acid-resistant P. Temperature: 37±0.1°C.

of phosphoglucomutase and phosphatase in the enzymic reaction mixture had practically no effect on the formation of G–1–P, although in the latter stage of the reaction, addition of magnesium or fluoride caused a marked change in the distribution pattern of phosphorus compounds. In the early stage, the formation of acid-resistant P was negligibly small. This showed that phosphorylase competed successfully with phosphoglucomutase for G–1–P, and, as a consequence, the latter activity was inhibited by the former, at least when the amount of G–1–P formed was small.

Therefore, irrespective of many disadvantages[17], the present procedure may be utilized for the determination of phosphorylase activity even in the presence of phosphoglucomutase and phosphatase activities.

DISCUSSION

Studies on phosphatases from plant materials are rather meager in the literature (see Roberts[18] for a short review on this subject). Such studies show that plant phosphatases are a group of isodynamic enzymes with different specificities towards substrates, activation by magnesium ions, and inhibition by fluoride. In the absence of a direct evidence for the presence of a phosphatase hydrolyzing G–1–P directly, it is not guaranteed that sodium fluoride effectively inhibits the phosphatase acting on G–1–P.

For the purpose of inhibiting phosphatase activities contaminating phosphorylase preparations, ammonium molybdate and sodium fluoride have been used so far. Thus, Bailey et al.[19] reported that 1.02×10^{-4} M molybdate completely inhibited phosphatase contaminant in their purified phosphorylase preparation, while Porter[3] used 0.02 M fluoride to inhibit phosphatase activity in her rather crude phosphorylase preparation from potatoes. In his work on the phosphorylase activities of corn seeds, Fuwa[20] used either 5×10^{-3} M ammonium molybdate or 0.02 M sodium fluoride. As partially purified potato phosphorylase is not affected by sodium fluoride in such a high concentration as 0.05 M[21], it may be assumed that fluoride at the concentration of 0.02 M has no effect on phosphorylases from other plant sources. However, the above authors did not give evidence that phosphatase activity was actually inhibited by fluoride or molybdate.

It was shown in this paper that sodium fluoride (0.02 M) did not inhibit completely phosphatases acting on G–6–P and glycerophosphate. However, it was shown that hydrolysis of G–1–P due to phosphatase action was very small, if ever present, in crude extract of potatoes. Even in extract of broad bean seeds, in which phosphoglucomutase activity was very high, the interference by the phosphoglucomutase-phosphatase system on the determination of phosphorylase activity was not so great as was anticipated. In crude extracts from leaves of some plant materials, the contribution by phosphatase on the liberation of P*i* often reached 10% and sometimes 20%. Therefore, the use of fluoride, although not completely satisfactory, is to be recommended in the determination of phosphorylase activities in crude extracts from plant materials.

Effects of enzymes other than phosphoglucomutase which act on G–1–P by converting it into other phosphorus compounds, e.g., transphosphatase and uridyl transferase, and the extent to which phosphatase activity is inhibited by molybdate are to be elucidated in a future study.

Acknowledgment The author wishes to express his sincere thanks to Prof. S. Funahashi for his interest in this work, and to Prof. M. Itô, Tokyo University of Agriculture and Technology, for his generous gift of β-amylase. Thanks are also due to Prof. S. Kawamura, Kagawa University, and Drs. H. Fuwa and T. Akazawa, Nagoya University, for their kind advice during the preparation of manuscript.

---

[17] M. Nakamura, in S. Akabori (ed.), *Kôso Kenkyu-hô* (Methods in Enzymology), Vol. II, p. 569. Asakura Shoten (Tokyo, 1956).

[18] D. W. A. Roberts, *J. Biol. Chem.*, **219**, 711 (1956).

[19] J. M. Bailey, G. J. Thomas and W. J. Whelan, *Biochem. J.*, **49**, lvi (1951).

[20] H. Fuwa, *Arch. Biochem. Biophys.*, **70**, 157 (1957).

[21] M. Nakamura, *Nippon Nôgei-kagaku Kaishi*, **26**, 267 (1952).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, 389~397, 1959]

Quantitative Analysis of Sugars in Plant Extracts by Ion-exchange Chromatography, with Special Reference to the Examination of Conditions for Preparing the Sample Sugar Solutions

By Kenji Mori and Michinori Nakamura

Department of Agricultural Chemistry, the University of Tokyo, Bunkyo-ku, Tokyo

Received December 16, 1958

Critical examinations were made on the conditions for preparing the sugar solutions to be analyzed by ion-exchange chromatography of sugar-borate complexes by the method of Khym and Zill1). A procedure was proposed which gave the best recovery of sugars with minimum hydrolysis of sucrose. By means of this procedure, sugar solutions were prepared from potato tubers which had been stored at a high (30°C) or low temperature (6°C). Results of the chromatographic separation and determination of component sugars showed that main sugars present in potato tubers were sucrose, glucose, and fructose. Maltose and pentoses could not be detected. The contents of sucrose, glucose, and especially, fructose were far greater in potatoes stored at a low temperature than in those stored at a high temperature.

Khym and Zill1) showed that borate complexes of sugars could be separated by ion-exchange chromatography. Since then, there appeared some papers on the analysis of sugars in plant tissues by the method. It was confirmed that essentially quantitative results could be obtained by the method of Khym and Zill and the accuracy of the method fairly surpassed that of paper chromatography.

While considerable attention has been paid to the separation of sugars, there is a few investigations of the procedures for preparing sugar solutions from plant tissues.

The procedures which have been used are as follows2,3,4): Plant tissues are extracted with hot 80% ethanol. The alcohol is removed in vacuo. Lead acetate solution is used to clean the extract and the excess lead removed by hydrogen sulfide. Deionization of the extract is accomplished by the use of ion-exchangers. The deionized solution is used for chromatography.

In these procedures, there are two points to be made clear. The first point concerns the problem whether the recovery of sugars is completely quantitative in these procedures. Loss of sugar may occur during removal of non-sugar components (i.e., during treatment with lead and removal of excess lead) and/or interfering ions by ion-exchangers. There are some reports concerning the adsorption of sugars by ion-exchangers. For example, strong-base anion-exchangers such as Amberlite IRA-400 or Dowex-1 (in hydroxide forms) adsorb and retain sugars, especially reducing sugars such as glucose and fructose5). Even a weak-base anion-exchanger such as Amberlite IR-4B (in hydroxide form) is also reported to adsorb glucose6), although it is not certain whether the amount of sugar adsorbed is beyond the analytical error or not.

---

1) J. X. Khym and L. P. Zill, *J. Am. Chem. Soc.*, **74**, 2090 (1952).

2) G. R. Noggle and R. A. Bolomey, *Plant Physiol.*, **26**, 174 (1951).

3) G. R. Noggle and L. P. Zill, *Arch. Biochem. Biophys.*, **41**, 21 (1952).

4) T. Fukui and Z. Nikuni, *Nippon Nôgei-kagaku Kaishi* (J. Agr. Chem. Soc. Japan), **33**, 72 (1959).

5) S. Roseman, R. H. Abeles and A. Dorfman, *Arch. Biochem. Biophys.*, **36**, 232 (1952).

6) T. M. Reynolds, *Nature*, **175**, 46 (1955).

Anyway, it is quite possible that adsorption of sugars can occur during the deionization with ion-exchangers.

The second point concerns the possibility that hydrolysis of sucrose may occur during these procedures. Use of hydrogen sulfide and cation-exchangers in the removal of lead ions and the other cations, respectively, makes the sugar solution very acid, whereby hydrolysis of sucrose may occur. In fact, Noggle and Bolomey[2] found that hydrolysis of sucrose ensued by strong-acid cation-exchangers (e.g., Dowex-50) during deionization. Hydrolysis of sucrose will cause a serious error in the analysis of sugars, especially when the plant tissue contains a large amount of sucrose and very little of glucose and/or fructose, as in the case of potato tubers stored for some time at a high temperature.

This paper deals with the examination of conditions for preparing the sugar solutions to be analyzed by ion-exchange chromatography of sugar-borate complexes. A procedure was proposed which gave the best recovery of sugars from plant tissues. By using the procedure, sugar contents of potatoes stored under different temperature conditions were determined.

EXPERIMENTAL

Materials and Methods

Sugars. D-Arabinose was prepared from calcium D-gluconate by the method of Hockett and Hudson[7]. Glucose, fructose, and sucrose were commercial products of the highest qualities.

Determination of Sugars. Total sugar was determined by the phenol method of Dubois et al.[8]. Reducing sugars were determined by the colorimetric method of Somogyi[9]. Pentose was determined by the orcinol method of Drury[10]. Fructose was determined by the resorcinol method of Roe[11].

Chromatography of Sugars. The preparation of an analytical column of Dowex-1 (200~400 mesh, borate form), 0.87 sq. cm. × 4.0 cm., and the chromatography of sugar-borate complexes on this column were performed just as described by Khym and Zill[1].

RESULTS AND DISCUSSION

Adsorption of Sugars by Ion-exchange Resins

As the desalting is usually performed with ion-exchange resins either in the form of columns or in batchwise fashion, these two procedures were examined for possible adsorption of sugars. In the column method, the following resins were used:

(i) Amberlite IR-120 (H^+), 2.5 sq. cm. × 5 cm.

(ii) Amberlite IR-4B (OH^-), 2.5 sq. cm. × 5 cm.

(iii) Amberlite IRA-410 (OH^-), 2.5 sq. cm. × 5 cm.

(iv) Mixed resins, containing 12 ml.* each of Amberlite IR-120 (H^+) and Amberlite IR-4B (OH^-). A column with an inner diam. of 1.8 cm. was used.

(v) Column (i) was followed by column (ii), so that the effluent from column (i) entered directly into column (ii).

One hundred ml. of a sugar solution (containing 200 mg. of sucrose, 100 mg. of glucose, or 100 mg. of fructose) was passed through one of the columns described above at a flow-rate of 2 ml. per min. After passage of the sugar solution, the column was washed with water until no sugar was detected in the effluent solution. The effluent solution and the washings were fractionated into 8-ml. portions by using a fraction-collector. Content of the sugar in each fraction was determined by the phenol method. A part of the results is shown in Fig. 1.

As shown in the figure, Amberlite IRA-410, a strong-base anion-exchange resin, retained glucose so firmly that a large amount of water was necessary to wash it out from the column. On the contrary, adsorption of sucrose was very slight with IR-120 and IR-4B resins. With these resins, recoveries of sucrose as well as of glucose

---

[7] R. C. Hockett and C. S. Hudson, *J. Am. Chem. Soc.*, **56**, 1672 (1934).

[8] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

[9] M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).

[10] H. Drury, *Arch. Biochem.*, **19**, 455 (1948).

[11] J. H. Roe, *J. Biol. Chem.*, **107**, 15 (1934)

* The volume of the resin refers to the wet volume throughout this paper.

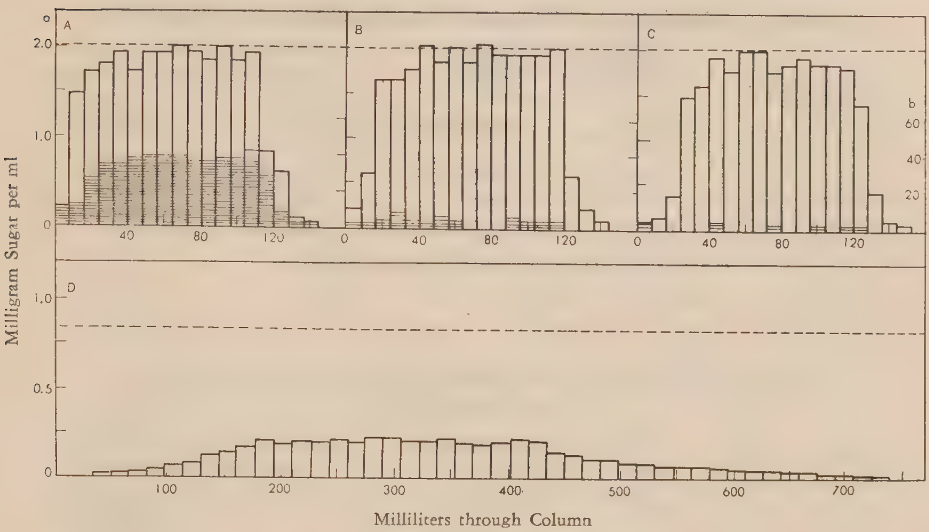


FIG. 1. Adsorption of Sugar by Strong-base Anion-exchangers and Hydrolysis of Sucrose by Strong-acid Cation-exchangers.

A broken line in the upper part of each diagram shows the original concentration of the sugar solution added to the column. The shadded area shows the amount of reducing sugars.

Ordinate: a. concentration of total sugar in mg./ml.
b. concentration of reducing sugar in μg./ml.
Abscissa: volume of effluent run out of column.
Flow rate: 2 ml./min.
A: Amberlite IR-120 (H^+), 2.5 sq.cm.×5 cm. Temperature: 28°. Recovery of sucrose, 102.9%; hydrolysis of sucrose, 2.0%.
B: Amberlite IR-4B (OH^-), 2.5 sq.cm.×6 cm. Temperature: 28°. Recovery of sucrose, 100.6%; hydrolysis of sucrose, 0.19%.
C: Vertically-connected columns of Amberlite IR-120 (H^+) (2.5 sq.cm.×5 cm.) and Amberlite IR-4B (OH^-) (2.5 sq.cm.×6 cm.). Temperature: 6°. Recovery of sucrose, 104.0%; hydrolysis of sucrose, 0.05%.
D: Amberlite IRA-410 (OH^-), 2.5 sq.cm.×6 cm. Temperature: 28°. Recovery of glucose, 100.1%.

and fructose always amounted to 98% or more of the sugar added to the columns, when these columns were washed with an equal volume of water after passage of the sugar solution.

In the batchwise method, it was at first necessary to know how long the sugar solution should be let to stand in contact with the resin to effect complete desalting. Williams et al.[12] allowed a suspension of 2 g. (dry wt.) each of anion and cation exchangers in 50 ml. of a sugar solution to stand for 2 hrs. with occasional shaking at every ten min., while Jones[13] agitated the same suspension for 15 min. Therefore, the time of contact of a solution with the resins necessary for complete desalting was determined by using a solution of mixed inorganic salts.

A mixture of 5 ml. each of IR-120 (H^+) and IR-4B (OH^-) resins was suspended in 50 ml. of a salt solution containing 100 mg. of potassium dihydrogen phosphate and 10 mg. each of calcium chloride dihydrate, magnesium chloride hexahydrate, and sodium chloride*. The suspension was cooled in an ice-water bath with occasional shaking. Before and after shaking for 5, 15, 30, and 60 min., aliquots were withdrawn and orthophosphate contents were determined by the method of Nakamura[14]. At the same time, specific resistance of the treated solution was

---

[12] K. T. Williams, A. Bevenue and B. Washauer, *J. Assoc. Offic. Agr. Chemists*, **33**, 980 (1950); cited by D. J. Bell, in K. Paech and M. V. Tracey (eds.), Modern Methods of Plant Analysis, Vol. II, p. 5. Springer Verlag (Berlin, 1955).

[13] N. R. Jones, *Biochem. J.*, **68**, 704 (1958).

* The solution contains the maximum amount of salts which would be extracted from potatoes by the method which will be given later in this paper.

[14] M. Nakamura, *Nippon Nôgei-kagaku Kaishi*, **24**, 1 (1950).

TABLE I. DESALTING ABILITIES OF ION-EXCHANGERS USED BATCHWISE

Resins used	Amberlite IR–120 (H^+)+ Amberlite IR–4B (OH^-)		Amberlite IR–120 (H^+)+ Amberlite IRA–410 (OH^-)	
	Specific resistance	Phosphorus	Specific resistance	Phosphorus
	ohm. cm	μg/ml	ohm. cm	μg/ml
Original solution	0.0×10^4	460	0.0×10^4	460
After 5 min.	1.0	21.4	0.6	23.7
〃 15 min.	8.2	0.4	4	1.6
〃 30 min.	10	0.0	12	0.0
〃 60 min.	18	—	22	—

TABLE II. RECOVERY OF GLUCOSE FROM ION-EXCHANGE RESINS USED BATCHWISE

Resins used	Amberlite IR–120 (H^+)+ Amberlite IR–4B (OH^-)		Amberlite IR–120 (H^+)+ Amberlite IRA–410 (OH^-)	
	mg	percentage recovered	mg	percentage recovered
Original solution	20.0	100	20.0	100
Filtrate	18.4	92	12.3	61.5
1st washing	1.8	9	3.1	15.5
2nd 〃	0.2	1	1.6	8.0
3rd 〃	0.0	0	0.8	4.0
4th 〃	—	—	0.5	2.5
Sum	20.4	102	18.3	91.5

measured with a conductometer (manufactured by Japan Organo Co.). Results are shown in Table I. It may be concluded from these results that desalting is nearly complete after 15 to 30 min. at the ice-cold temperature.

The next problem in the batchwise use of resins is the adsorption of sugars on the resins. A mixture of 2 ml. each of IR–120 and IR–4B was added to 20 ml. of a solution containing 20 mg. of glucose. The suspension was stirred for 30 min. in an ice-water bath, and then the resin was filtered off with a glass-filter. The resin was washed four times with 20-ml. portions of water. Glucose contents in the filtrate and washings were determined separately. The same procedure was repeated by using IRA–410 resin instead of IR–4B. The results are shown in Table II. When IR–4B was used in combination with IR–120, recovery of glucose was complete after the second washing. IRA–410 resin retained glucose very firmly even in the batchwise method just as it did in the column method.

Recovery of Sugars from Analytical Columns

Although it was reported that sugars were eluted quantitatively form borate-form resins[1,3,4], it was desirable to confirm the quantitative aspect in our hands. An analytical column, 0.87 sq. cm.×4 cm., and a mixture of authentic sugars, containing 20.0 mg. of sucrose, 4.0 mg. of glucose, and 4.0 mg. of fructose per 10 ml. were prepared as described by Khym and Zill[1]. The recovery of each sugar was 19.8 mg. (99%), 3.7 mg. (93%), and 4.0 mg. (100%), respectively.

Hydrolysis of Sucrose

It is possible that hydrolysis of sucrose may occur when the sugar solution is exposed to strong acid, i.e., during removal of lead ions by hydrogen sulfide and during desalting with a strong-acid cation-exchanger. To confirm the extent to which hydrolysis of sucrose may occur

TABLE III. HYDROLYSIS OF SUCROSE DURING REMOVAL OF LEAD IONS

	Original solution	After treating with H_2S	After treating with Na_2HPO_4
Total sugar as sucrose, mg	190	190	190
Reducing sugar as glucose, mg	0.30	0.72	0.27
Percentage hydrolysis	—	0.22	0.0

TABLE IV. HYDROLYSIS OF SUCROSE DURING DESALTING WITH ION-EXCHANGE RESINS USED IN COLUMNS

Resins used	IR–120		IR–4B	IR–120 + IR–4B Mixed bed		IR–120 + IR–4B Separated
Temperature	6°	28°	28°	6°	28°	6°
Total sugar as sucrose, mg	200	200	200	200	200	200
Reducing sugar as glucose, mg	0.18	3.90	0.38	0.05	2.10	0.10
Percentage hydrolysis	0.09	2.0	0.19	0.03	1.1	0.05

and to find out, if possible, a mean by which the hydrolysis may be prevented, the following experiments were performed.

Hydrolysis of Sucrose during Removal of Lead Ions

Although hydrogen sulfide has been used for the removal of lead ions, there are some reports in which sodium phosphate is used for the same purpose. It was attempted, therefore, to decide which one of these two reagents might cause a smaller hydrolysis of sucrose.

One ml. of lead acetate solution (containing 0.3 g. of $Pb(CH_3COO)_2 \cdot 3H_2O$) was added to 50 ml. of a solution containing 190 mg. of sucrose. After cooling the solution for 30 min. in ice-water, hydrogen sulfide was passed through the solution for 5 min. Lead sulfide thus formed was centrifuged off and washed with three portions of cold water saturated with hydrogen sulfide. All these procedures were performed within 30 min. The combined supernatant and washings were aerated for 30 min. at room temperature to set free the solution from excess of hydrogen sulfide. The total and reducing sugar contents in the solution were determined. In another experiment, 2 ml. of sodium phosphate solution (containing 0.2 g. of $Na_2HPO_4 \cdot 12 H_2O$ per ml.) was added to the sugar solution containing lead acetate. The precipitate formed was centrifuged off and washed with three portions of cold water. The combined supernatant and washings were used for the determination of total and reducing sugar contents.

The results are shown in Table III. Hydrolysis of sucrose occurred when the sugar solution was treated with hydrogen sulfide, although it was limited only to a small extent when due precaution was paid to keep the sugar solution well cooled during treatment with hydrogen sulfide. It may be, however, better to remove lead ions with sodium phosphate in order to avoid the hydrolysis of sucrose.

Hydrolysis of Sucrose during Desalting

In the experiments in which adsorption of sugars on the resin was determined, the extent of the hydrolysis of sucrose was determined at the same time. The appearance of reducing sugars in the eluates from the column is also shown in Fig. 1. Table IV shows the extents of hydrolysis of sucrose by various resins used in columns at room and cold-room temperatures. Treatment with IR–120 resin brought about

TABLE V. HYDROLYSIS OF SUCROSE DURING DESALTING WITH ION-EXCHANCE RESINS USED BATCHWISE

Temperature	3°			28°		
Period of shaking, min.	0	15	30	0	15	30
Total sugar as sucrose, mg	37.6	37.6	37.6	37.6	37.6	37.6
Reducing sugar as glucose, mg	0.08	0.12	0.16	0.08	0.21	0.34
Percentage hydrolysis	0.20	0.31	0.40	0.20	0.54	0.86

the hydrolysis of sucrose to a considerable degree at room temperature; about 2% of sucrose was hydrolyzed under these conditions. This may cause a serious error in the analysis of sugars. However, hydrolysis was remarkably suppressed by carrying out the whole procedure in a cold room at 6°. Hydrolysis of sucrose was much smaller when IR–120 resin was used in a mixed-bed form with IR–4B resin than when these resins were used separately. As was expected, IR–4B resin did not cause hydrolysis of sucrose to a significant degree.

In the batchwise method, 2 ml. each of IR–120 and IR–4B resins were added to 20 ml. of a sugar solution containing 37.6 mg. of sucrose. The suspension was stirred at room temperature or at ice-water temperature. Total and reducing sugars were determined before and after stirring for 15 and 30 min., respectively. The results are shown in Table V. Although less sucrose was hydrolyzed than the case in which resins were used in columns, a considerable amount of sucrose was hydrolyzed when the batchwise procedure was carried out at a high temperature. When the procedure was performed in ice-water, the extent of hydrolysis of sucrose was very small, i.e., 0.2% of sucrose present in the sugar solution.

Recovery of Added Arabinose from Potato Extract

The experiments described so far were based on the principle that interfering substances in tissue extracts were removed by lead ions, excess lead was removed by hyrogen sulfide or sodium phosphate, and further desalting was carried out with ion-exchange resins. Then, a question arises: Is the removal of lead ions prior to desalting actually necessary? It was to be expected that excess lead ions might be removed in the desalting process. To test the validity of the idea on one hand and to check the quantitative aspect of the whole procedure on the other, the following experiments were performed.

Potato tubers were extracted with hot 80% ethanol[15]. Since the virtual absence of pentoses in potato extracts was already known from paper-chromatographic analysis, a small amount of arabinose was added to the extract as a reference sugar. After removal of ethanol by distillation in vacuo, the solution was treated with lead acetate to remove interfering substances. Sugar solutions to be analyzed by ion-exchange chromatography were prepared by one of the following three methods:

(1) Lead ions were removed by hydrogen sulfide in the cold. After desalting with a mixture of IR–120 and IR–4B by the batchwise method, the solution was concentrated in vacuo.

(2) Lead ions were removed with sodium phosphate. Other procedures were the same as in (1).

(3) The solution containing excess lead ions was treated with ion-exchangers, and the resulting deionized solution was concentrated in vacuo.

Ion-exchange chromatography of the resulting solutions was carried out as described by Khym and Zill[1]. The amounts of sucrose, glucose, fructose, and arabinose eluted from the column as well as the percentage recovery of added

---

[15] K. T. Williams, E.F. Potter, A. Bevenue and W. R. Scurgi, *J. Assoc. Offic. Agr. Chemists*, **32**, 698 (1949); cited by D. J. Bell, loc. cit., p. 3.

TABLE VI. RECOVERY EXPERIMENTS USING POTATO EXTRACT

In each experiment, 50.0 ml aliquot of potato extract, to which 5.35 mg of arabinose had been added, was used.

Method used to remove lead ions	Hydrogen sulfide	Sodium phosphate	Ion-exchange resins
Sucrose, mg	26.6	25.8	25.6
Fructose, mg	3.8	4.3	4.0
Arabinose, mg	4.9 (92%)*	5.2 (97%)*	5.1 (95%)*
Glucose, mg	5.6	5.8	5.9

* Figures in parentheses show the percentage recovery of added arabinose.

arabinose are shown in Table VI. As will be seen from the table, the three methods gave essentially the same results. Thus, the yield of each sugar and the recovery of added arabinose might be within experimental error of this method. The elution pattern obtained with a sugar solution prepared by the method (1) is shown in Fig. 2 as an example. The results obtained here lead to the conclusion that the simplest method, i.e., the method (3), is the prefered one for preparing the sample sugar solutions.

Quantitative Analysis of Sugars in Potato Tubers
Potato tubers (var. Irish cobbler or Danshakuimo) were stored either at 6°C for 14 days or at 30°C for 24 days, respectively. Fifty g. of peeled potatoes were mashed into 200 ml. of 95% ethanol containing a small amount of calcium carbonate, and the mixture was refluxed for an hour. Four successive extractions were made each time with 70 ml. portions of hot 80% ethanol. The extracts were combined, and the volume was measured. A portion of the air-dried residue was hydrolyzed with dilute hydrochloric acid and the starch content of the potato tubers was calculated from the amount of reducing sugars thus formed.

An aliquot of the extract was concentrated in vacuo to about 10 ml. at a temperature not exceeding 40°. The concentrated solution was transferred quantitatively to a centrifuge tube by means of a small amount of water, and the solution was treated with 0.4 ml. of a lead acetate solution (0.3 g. $Pb(CH_3COO)_2 \cdot 3H_2O$/ml.). The precipitate was centrifuged off and washed with three portions of water. The combined supernatant and washings were treated with a mixture of 3 ml. each of IR–120 and IR–4B.

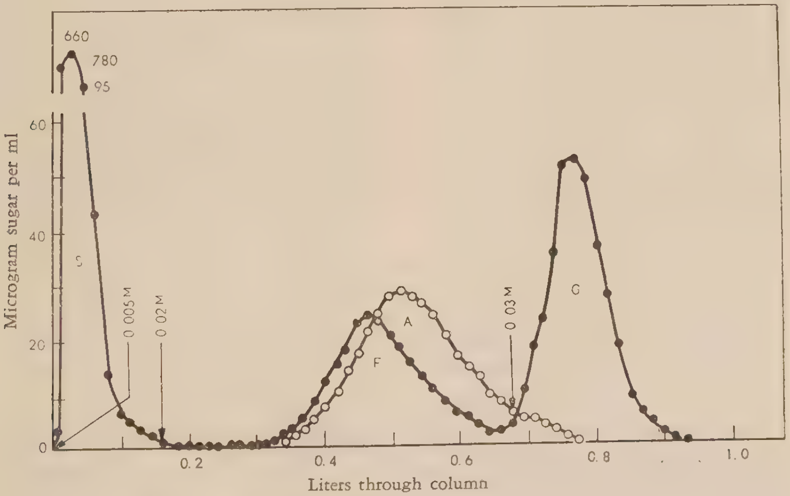


FIG. 2. Separation and Analysis of Sugars Extracted from Potato Tubers with Added Arabinose.

See the text for the preparation of the sample sugar solution. Concentrations of sodium tetraborate in the eluting solvents are shown in the figure. Flow rate: 1 ml./min. See Table VI for the amounts of sugars found in the eluates. The open circle shows the concentration of arabinose. Abbreviations used in the figure: S, sucrose; F, fructose; A, arabinose; and G, glucose.

The suspension was stirred for 20 min. in the cold, and the resin was filtered off on a glass-filter. The combined filtrate and washings were concentrated in vacuo. Total and reducing sugars were determined in the concentrated solution. An appropriate amount of 0.005 M sodium tetraborate solution was added to the concentrated solution to give a final concentration of 0.001 M. Preparation of an analytical column of Dowex-1, borate form, and chromatography of sugars on this column were performed as described by Khym and Zill[1].

Dubois' phenol method was used for the determination of total sugars. Although the

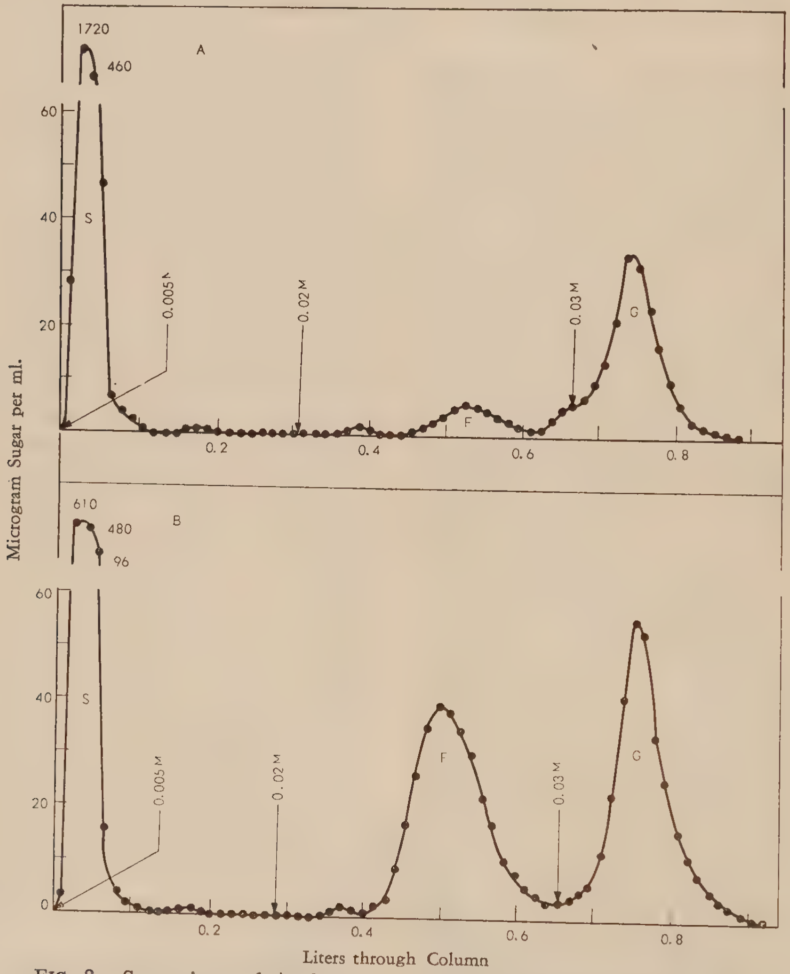


FIG. 3. Separation and Analysis of Sugars Extracted from Potato Tubers.

See the text for experimental details. Concentrations of sodium tetraborate in the eluting solvents are shown in the figure. Flow rate: 1.5 ml./min.

A: Potato tubers were stored at 30° for 24 days. The sugar solution, corresponding to 11.4 g. of fresh potato tubers, was put on the column. Sugars found: 31.2 mg. of sucrose; 0.56 mg. of fructose; and 2.7 mg. of glucose.

B: Potato tubers were stored at 6° for 14 days. The sugar solution, corresponding to 1.7 g. of fresh potato tubers, was put on the column. Sugars found: 19.5 mg. of sucrose; 4.3 mg. of fructose; and 4.3 mg. of glucose.

See the legend of Fig. 2 for abbreviations in the figure.

development of color in this method was slightly inhibited in the presence of borate, the proportionality between color intensity and concentration of sugar was maintained even in the presence of borate. Somogyi's colorimetric method⁹⁾ could not be used for the determination of reducing sugars eluted by 0.03 M borate, because development of color was seriously inhibited by borate. Roe's resorcinol method for fructose¹¹⁾ was not affected by borate.

The results are shown in Fig. 3. These results are in fairly good agreement with those calculated from chemical determinations. Thus, 11.4 g. of potatoes stored at 30° contained 34.5 mg. of total sugars and 3.3 mg of reducing sugars by the chromatographic method, while corresponding values obtained by chemical methods were 35.0 and and 4.5 mg., respectively. Total and reducing sugars contained in potatoes stored at 6° (1.7 g. fresh wt.) were 28.1 and 8.6 mg., respectively, by the chromatographic method, and 25.1 and 9.7 mg., respectively, by the chemical method. As can be seen in the figure, maltose could not be found in the eluates. A peak following that of sucrose seems to correspond with raffinose and a peak preceding that of fructose seems to correspond with stachyose, as judged from chromatographic behaviors of these sugars. But the sugars constituting these peaks were not identified in this work, because the amounts of these sugars obtained were so small that it was difficult even for a tentative identification by paper chromatography.

TABLE VII. CARBOHYDRATE CONTENTS OF POTATO TUBERS AS INFLUENCED BY STORAGE TEMPERATURES

Figures show the percentage contents in fresh potato tubers. See the text and the legend of Fig. 3 for experimental details.

Temperature of storage	6°*	30°**
Starch	12.0	11.3
Sugars	1.62	0.31
Sucrose	1.12	0.28
Glucose	0.25	0.024
Fructose	0.25	0.005

* Stored for 14 days.
** Stored for 24 days.

Table VII shows the percentage composition of sugars and starch in the fresh potato tubers. As shown in the table, the sugar contents of potatoes stored at a lower temperature were much greater than those of potatoes stored at a higher temperature. Especially, the increase in the amount of fructose in the cold was remarkably great. These results were essentially the same as those obtained by Arreguin-Lozano and Bonner¹⁶⁾ and by Schwimmer et al.¹⁷⁾, but the changes in the contents of glucose and fructose were far greater than those reported by these workers.

Acknowledgment We wish to express our sincere thanks to Prof. S. Funahashi for his interest in this work.

---

16) B. Arreguin-Lozano and J. Bonner, *Plant Physiol.*, **24**, 720 (1949).

17) S. Schwimmer, A. Bevenue, W. J. Weston and A. L. Potter, *J. Agr. Food Chem.*, **2**, 1284 (1954).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 398～405, 1959]

The Sucrose-synthesizing Enzyme in the Beans

By Michinori NAKAMURA

Department of Agricultural Chemistry, the University of Tokyo, Bunkyo-ku, Tokyo

Received January 19, 1959

The properties of UDPG → fructose transglucosylase, partially purified from immature soybeans or broad beans, were investigated. Magnesium ions had a stimulating effect on this enzyme. Evidence was presented to show that UDPG, the glucosyl group donor for sucrose synthesis, was regenerated from UDP and G–1–P in the presence of ATP.

Hassid et al.[1] reported that sucrose was synthesized by an enzyme preparation obtained from *Pseudomonas saccharophila* by the following reaction:

$$\text{G–1–P}^{*}+\text{fructose} \rightleftharpoons \text{sucrose}+\text{P}_i$$

This enzyme, which was named sucrose phosphorylase, has not been found in plant tissues irrespective of much effort of many investigators and the enzymic mechanism by which sucrose is synthesized has remained obscure for a long time[2].

Buchanan et al.[3] showed that, after a short time exposure of Chlorella and Scenedesmus to $C^{14}O_2$ in the light, UDPG and sucrose phosphate as well as hexose monophosphates were rapidly labeled. Sucrose phosphate, with its phosphate group attached to carbon–1 of the fructose moiety, was subsequently isolated from sugar beet leaves[4]. These evidences led Buchanan to assume that sucrose is synthesized in plants by the following reactions:

$$\text{UDPG}+\text{fructose-1-phosphate}\rightarrow \text{sucrose phosphate}+\text{UDP}$$

$$\text{Sucrose phosphate}+H_2O\rightarrow\text{sucrose}+Pi$$

A short time later, Leloir and Cardini[5] discovered an enzyme in wheat germ and some other plant materials which catalyzed sucrose synthesis stoichiometrically according to the following reaction:

$$\text{UDPG}+\text{fructose} \rightleftharpoons \text{sucrose}+\text{UDP} \quad (1)$$

Later, they found that wheat germ contained, besides this enzyme[6] (UDPG→fructose transglucosylase), another enzyme[7] which catalyzed synthesis of sucrose phosphate according to the following reaction:

$$\text{UDPG}+\text{F–6–P}\rightleftharpoons\text{sucrose phosphate}+\text{UDP} \quad (2)$$

Sucrose phosphate, obtained by reaction (2), had its phosphate group attached to carbon–6 of the fructose moiety and thus differed from a similar compound obtained by Buchanan from sugar beet leaves.

Almost at the same time and independently from the Leloir group, Turner[8] obtained an enzyme preparation from peas which synthesized sucrose from G–1–P and fructose. Sucrose synthesis was much stimulated by the addition to this system of a nucleotide fraction from yeasts along with magnesium ions[9]. The reaction (1) discovered by Leloir and Cardini was assumed to be responsible for the final step in a complicated reaction sequence leading to sucrose synthesis in that pea enzyme preparation[6,10].

---

* The following abbreviations are used: G-1-P, α-D-glucose-1-phosphate; F-6-P, fructose-6-phosphate; UDPG, uridine diphosphate glucose; UTP, uridine triphosphate; UDP, uridine diphosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; *Pi*, inorganic orthophosphate; PP, inorganic pyrophosphate; tris, tris (hydroxymethyl) aminomethane.

1) W. Z. Hassid and M. Doudoroff, *Advances in Enzymol.*, **10**, 123 (1950).

2) W. Z. Hassid and E. W. Putman, *Ann. Rev. Plant Physiol.*, **1**, 109 (1950).

3) J. G. Buchanan, J.A. Bassham, A.A. Benson, D. F. Bradley, M. Calvin, L.L. Daus, M. Goodman, P. M. Hayes, V. H. Lynch, L. T. Norris and A. T. Wilson, in W. D. McElroy and B. Glass (eds.), Phosphorus Metabolism, Vol. II, p. 440. The Johns Hopkins Press (Baltimore, 1952).

4) J. G. Buchanan, *Arch. Biochem. Biophys.*, **44**, 140 (1953).

5) L. F. Leloir and C. E. Cardini, *J. Am. Chem. Soc.*, **75**, 6084 (1953).

6) C. E. Cardini, L. F. Leloir and J. Chiriboga, *J. Biol. Chem.*, **214**, 149 (1955).

7) L. F. Leloir and C. E. Cardini, *J. Biol. Chem.*, **214**, 157 (1955).

8) J. F. Turner, *Nature*, **172**, 1149 (1953).

9) J. F. Turner, *Nature*, **174**, 692 (1954).

In view of the important role sucrose plays in the carbohydrate metabolism in plants, the author examined the distribution of UDPG→fructose transglucosylase in plants. It was found that soybeans and broad beans—especially when they were not yet mature—were rich sources of this enzyme. The properties of this enzyme, partially purified from these sources, are somewhat different from those of a similar enzyme from wheat germ[6] and are the main concern of this paper. Evidence indicating the mechanism for the regeneration of UDPG in plants is also included.

EXPERIMENTAL

Materials.

UDPG was prepared by the method of Caputto et al.[11] The final product was stored frozen at −20° or as the dried barium salt. Purity was assayed by the amount of acid-labile glucose (0.01 N sulfuric acid, 100°, 7 min.). ATP was prepared by the method of Dounce et al.[12] G-1-P was prepared as described previously[13]. F-6-P was prepared by the method of Neuberg et al.[14] Sugars and other reagents were commercial products.

Measurement of Enzymic Activity.

An assay system contained 100 μmoles of tris-maleate buffer of pH 7.4, 2.4 μmoles of UDPG, 12.5 μmoles of fructose, 7.5 μmoles of magnesium chloride and an appropriate amount of enzyme; total volume, 0.75 ml. All the components, except UDPG, were mixed and incubated at 37±0.1°C. The reaction was started by the addition of UDPG, also maintained at the same temperature. Two-tenth ml. each of the reaction mixture was withdrawn immediately after the start of the reaction and at 10 and 20 min. after the first sampling. The aliquot was treated with 1.3 ml. of water and 0.3 ml. of 2 N sodium hydroxide and the resulting mixture was heated in a boiling water bath for 10 min. in order to destroy any remaining free fructose. Sucrose content was determined by the method of Roe[15]. In earlier experiments, the reaction mixture was deproteinized by the method of Somogyi[16] and an aliquot of the deproteinized filtrate was used for the determination of sucrose. However, it was found that deproteinization procedure could be omitted without deleterious effect on subsequent colorimetry. In the determination of sucrose, appropriate sucrose standards were run at the same time. One unit was defined as the amount of enzyme catalyzing the formation of 1 μmole of sucrose in 10min. under the conditions given above.

TABLE I. PURIFICATION OF BROAD BEAN ENZYME

Fraction	Volume	Activity	Total activity	Protein content*	Specific activity
	ml	units/ml	units		
I. crude extract	150	0.54	81	0.89	0.62
II. 1st ammonium sulfate	75	0.60	45	0.34	1.77
III. manganous chloride**	70	—	—	—	—
IV. 2nd ammonium sulfate	50	0.43	22	0.10	4.30

* Protein content was determined by the biuret method of Gornall et al.[17] and was expressed as the optical density at 530 mμ at 1-cm light path per ml of the enzyme solution.

** Owing probably to incomplete centrifugation, a small amount of precipitate remained in the solution and interfered with the determinations. Therefore, results were not given in the table.

Preparation of Enzyme Solutions.

Seventy g. of immature soybeans or broad beans were blended with 170 ml. of 0.05 M phosphate buffer, pH 7.0. The pressed juice was centrifuged at 3000 r.p.m. for 5 min. The supernatant solution (Fraction I, crude extract) was fractionated with ammonium sulfate at pH 7.0. The precipitate obtained between 8∼30 g. ammonium sulfate per 100 ml. was dissolved in 70 ml. of water and dialyzed against distilled water. To the centrifuged solution (Fraction II), 0.1 vol. of 1 M manganous chloride was added and the suspension was stirred for 30 min. After centrifugation, a slightly turbid solution (Fraction III) was fractionated again with ammonium sulfate, the precipitate obtained between 12∼28 g. per 100 ml. being saved this time. The precipitate was dissolved in 30 ml. of distilled

---

10) J. F. Turner, *Biochem. J.*, **67**, 450 (1957).

11) R. Caputto, L. F. Leloir, C. E. Cardini and A. C. Paladini, *J. Biol. Chem.*, **184**, 333 (1950).

12) A. L. Dounce, A. Rothstein, G. T. Beyer, R. Meier and R. M. Freer, *J. Biol. Chem.*, **174**, 361 (1948).

13) K. Mori and M. Nakamura, This Bulletin, **23**, 272 (1959).

14) C. Neuberg, H. Lustig and M. A. Rothenberg, *Arch. Biochem.*, **3**, 33 (1943).

15) J. H. Roe, *J. Biol. Chem.*, **107**, 15 (1934).

16) M. Somogyi, *J. Biol. Chem.*, **160**, 69 (1945).

17) A. G. Gornall, C. J. Bardawill and M. M. David, *J. Biol. Chem.*, **177**, 751 (1949).

water and dialyzed against several changes of distilled water. After centrifugation, the supernatant fluid (Fraction IV) was stored frozen at −20° without significant loss in enzymic activity for several months. All the above procedures were carried out in the cold (4°C). An example of the purification procedure is shown in Table I. Although fractionation with ethanol yielded a fraction with higher specific activity, the yield was so poor that this procedure was not adopted.

RESULTS

Since results obtained with soybean or broad bean enzyme were essentially similar, those obtained with the former enzyme are given below with a few exceptions.

Relationship Between the Amount of Enzyme and Activity: Time courses of sucrose formation in the assay systems with various amount of enzyme are shown in Fig. 1. In the early stage of the reaction, the amount of sucrose formed per unit time was approximately proportional to the amount of enzyme. The reaction followed the zero order kinetics until approximately 20% of UDPG added to the system had been used up for sucrose formation. Except when the amount of enzyme was very small, the reaction reached apparent equilibrium when approximately 80% of UDPG added to the system was converted into sucrose.

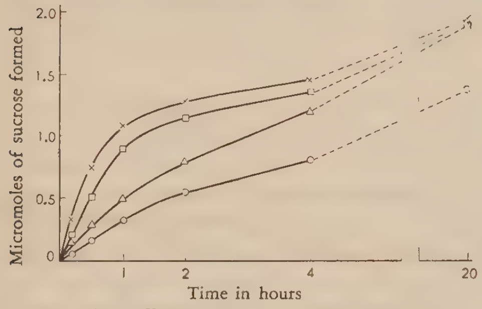


FIG. 1. Effect of the Amount of Enzyme.

System as described in the text. The amount of soybean enzyme added to each curve: ○, 0.01 ml.; △, 0.025 ml.; □, 0.05 ml.; ×, 0.10 ml.

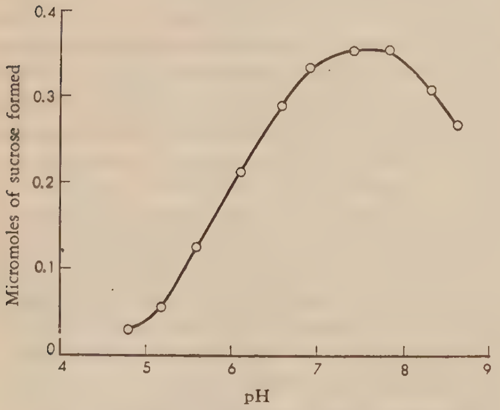


FIG. 2. Effect of pH on Enzymic Activity.

System as described in the text with tris-maleate buffer at the indicated pH value and at 0.133 M final concentration; 0.05 ml. of soybean enzyme. Incubated for 10 min. Average of duplicate experiments.

TABLE II. EFFECTS OF ADDED MAGNESIUM IONS AND MAGNESIUM-BINDING AGENTS ON ENZYME ACTIVITY

System as described in the text with variable amount of magnesium ions; 0.1 ml of soybean enzyme. Other additions are indicated in the table. Figures in the table represent µmoles of sucrose formed in 10 min.

Binding agents / Additions	Versene	Fluoride	Citrate
None	0.094	0.132	0.123
Binding agent, 15 µmoles	0.075	0.083	0.120
〃 〃, 7.5 µmoles	0.085	0.129	0.117
〃 〃, 7.5 µmoles + Mg++, 7.5 µmoles	0.212*	0.145	0.164
Mg++, 7.5 µmoles	0.181	0.225	0.211
〃, 15 µmoles	0.255**	0.251	0.284

* 15 µmoles of Mg++.
** 10 µmoles of Mg++.

TABLE III. EFFECTS OF VARIOUS DIVALENT METAL IONS ON ENZYMIC ACTIVITY

System as described in the text, except magnesium ions were replaced by various divalent metal ions as indicated in the table; 0.1 ml of soybean enzyme. Activity of the control reaction mixture without added metal ions was taken as 100. Concentration of metal ions was expressed as the final concentration.

Additions	Expt. 1. 0.01 M	Expt. 2. 0.02 M	Expt. 3. 0.01 M
None	100	100	100
Mg++	334	239	294
Ca++	285	168	
Mn++	273	189	
Ba++	85	75	
Fe++	81	118	
Zn++			24
Co++			228

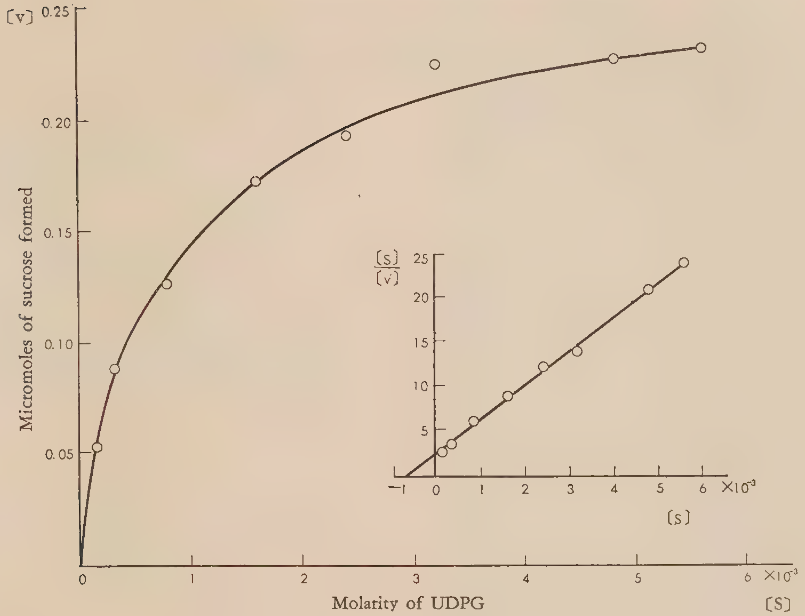


FIG. 3. Influence of UDPG Concentration.

System as described in the text; 0.05ml. of soybean enzyme. UDPG concentrations as indicated. Incubated for 10 min. Section at right, a Lineweaver-Burk[18] plot $Ks=0.73\times10^{-3}$.

Optimum pH: As shown in Fig. 2, the highest activity was obtained at pH 7.4∼7.8 with 0.133 M tris-maleate buffer.

Effect of Divalent Cations: As shown in Fig. 5 and Table II, the enzyme solution used in these experiments was active in the absence of added magnesium ions. However, addition of magsium ions in concentrations of 0.01∼0.02 M caused a great increase in the enzymic activity. Enzymic activity was suppressed by Versene* and fluoride, but not by citrate, and was reversed by magnesium. Effects of various divalent cations are shown in Table III. Calcium, magnesium, manganese and cobaltous ions had activating effects, while barium and zinc ions were inhibitory; ferrous ions were without effect.

Effect of Substrate Concentration: The results of experiments with different concentrations of UDPG or fructose are shown in Figs. 3 and 4, respectively. The calculated Michaelis constants were 0.73×10^{-3} M/l. for UDPG and 1.15×10^{-3} M/l. for fructose.

Specificity: As shown in Fig. 5, the enzyme solution used in this experiment was active with both fructose and F–6–P, although the activity with the latter was far lower than that with the former substrate. Addition of magnesium was also effective in enhancing the activity with F–6–P. In order to test the possibility that in the reaction with F–6–P the fructose liberated from F–6–P might be the true substrate of the enzyme, the phosphatase activity of the enzyme solution to hydrolyze F–6–P was measured. The results are shown in Fig. 6. The amount of fructose to be formed under the conditions of Fig. 5 would be sufficient to account for the measured activity with F–6–P in the figure.

Effect of Addition of G–1–P and ATP: The enzyme was tested whether it had the ability to convert G–1–P into UDPG. The results are shown in Fig. 7. When G–1–P along with ATP was added to the assay system, the amount of

---

[18] H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1935).

* Ethylenediamine tetraacetate.

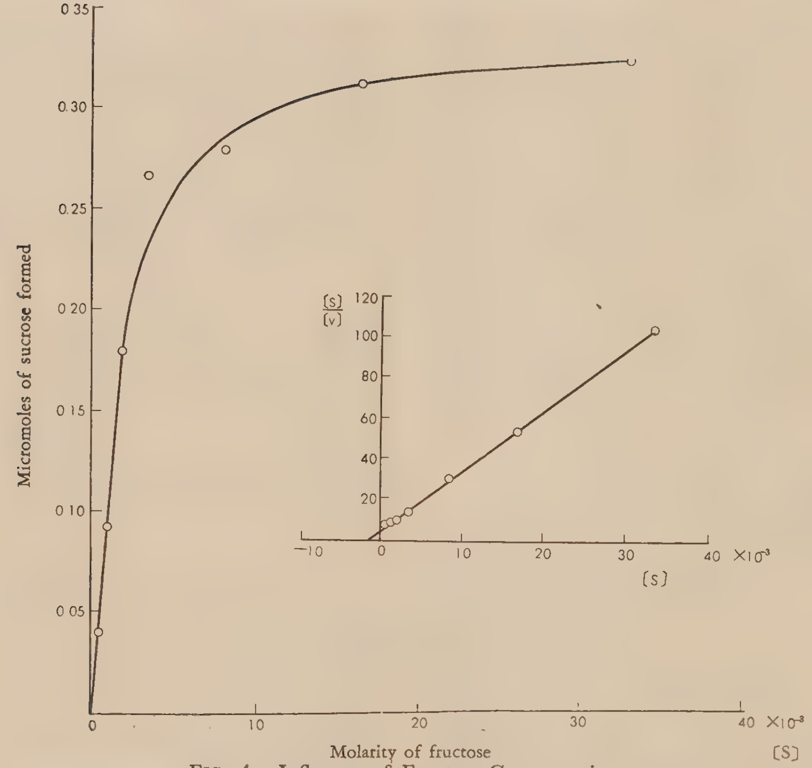


FIG. 4. Influence of Fructose Concentration.

System as described in the text; 0.1 ml. of soybean enzyme. Fructose concentrations as indicated. Incubated for 10 min. Section at right: a Lineweaver-Burk plot. $K_s = 1.15 \times 10^{-3}$.

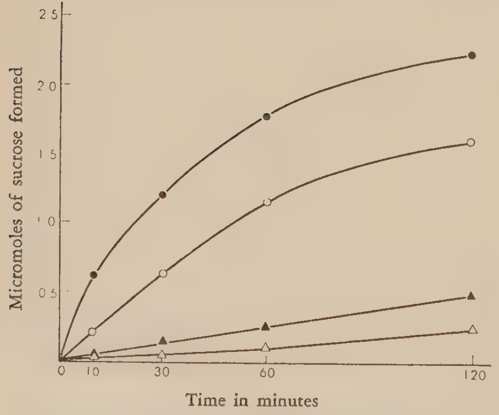


FIG. 5. Substrate Specificity and the Effect of Added Magnesium Ions.

Each reaction mixture contains, in a total volume of 1.2 ml., 160 μmoles of tris-maleate buffer of pH 7.4, 3.9μ moles of UDPG, and 0.1 ml. of soybean enzyme. Other additions are: ○, 16 μmoles of fructose; ●, 16 μmoles of fructose and 15 μmoles of magnesium chloride; △, 17 μmoles of F-6-P; ▲, 17 μmoles of F-6-P and 15 μ moles of magnesium chloride.

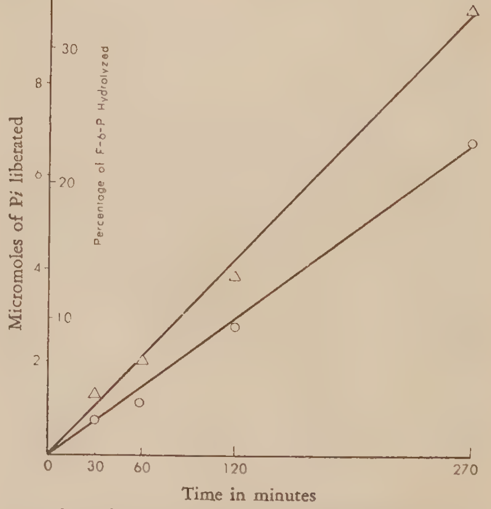


FIG. 6. Phosphatase Activity Hydrolyzing F-6-P.

Each reaction mixture contains, in a total volume of 1.6 ml., 200 μmoles of tris-maleate buffer of pH 7.4, 29 μ moles of F-6-P and 0.25 ml. of soybean enzyme. The curve represented by △ contains, in addition, 20 μmoles of magnesium chloride.

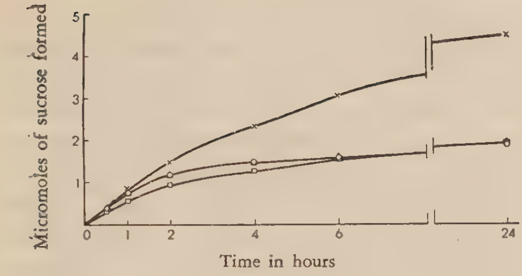


FIG. 7. Effect of Added G-1-P and ATP on Sucrose Synthesis.

System as described in the text; 0.05 ml. of soybean enzyme. Other additions are: △, 15 μmoles of G-1-P; □, 15 μmoles of ATP; ×, 15 μmoles each of G-1-P and ATP.

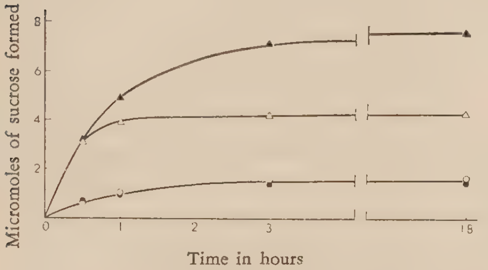


FIG. 8. Localization of the Enzyme in the Particulate and the Supernatant Fractions of Broad Beans.

Immature broad beans were blended with 2 vols. of ice-cold 0.3 M mannitol containing 0.033 M phosphate buffer, pH 7.0. The suspension was centrifuged at 3000 r. p. m. for 3 min. The supernatant fluid was centrifuged at 12,000×g. for 15 min. The sediment was washed twice with the homogenizing mixture and suspended in the same solution. The supernatant fluid was treated with ammonium sulfate (36 g./100 ml.) and the precipitate was taken in 0.033 M phosphate buffer, pH 7.0. Fractionation with ammonium sulfate was repeated once more. The solution thus obtained was dialyzed against running tap water for 2 hrs. and centrifuged at 12,000×g. for 15 min. All the operations were carried out in the cold.

Each reaction mixture contains, in a total volume of 3 ml., 150 μmoles of tris-maleate buffer, pH 7.4, 6 μmoles of UDPG, 25 μmoles of fructose and other ingredients as indicated below. Circles; the particulate fraction (corresponding to 1 g. of fresh broad beans). Triangles: the supernatant fraction (corresponding to 0.5g. of fresh broad beans). Closed signs: 20 μmoles of G-1-P and 25 μmoles of ATP.

sucrose synthesized was much increased and fairly surpassed the maximum expected amount of sucrose based on the amount of UDPG initially added to the system. Addition of either G–1–P or ATP alone had no effect on the amount of sucrose synthesized.

Localization of the Enzyme: The particulate and the supernatant fractions obtained from immature broad beans were tested for the enzymic activity. The results are shown in Fig. 8. Enzymic activity was found in both the particulate and the supernatant fractions, although the activity of the latter was much higher than that of the former. Moreover, it was also found that the ability to regenerate UDPG from G–1–P and ATP was absent in the particulate fraction.

Changes in Enzymic Activity during Germination: Results of experiments in which enzymic activity was followed during germination of mung beans are given in Table IV. The enzymic activity showed the lowest value at the 2nd day of germination and thereafter increased again.

TABLE IV. CHANGES IN ENZYMIC ACTIVITY DURING THE GERMINATION OF MUNG BEANS

Mung beans were germinated in the dark at 25°. Seedlings were blended with two vols. of 0.05 M phosphate buffer, pH 7.0. The suspension was centrifuged and the supernatant fluid was used as the enzyme solution. The assay system as described in the text.

Days of germination	Enzyme activity	Protein content*	Specific activity
	units/ml		
1.	0.74	0.84	0.88
2.	0.03	0.40	0.08
3.	0.11	0.30	0.37
4.	0.19	0.22	0.86

* See the legend of Table I.

DISCUSSION

Cardini et al.⁶⁾ reported that magnesium ions were without effect on this enzyme. The high concentration of magnesium (0.05 M) used by these workers might possibly be responsible for their failure to find the activating effect of this ion. In the author's experiments, the wheat germ enzyme, partially purified according to the method given in this paper, responded to magnesium in a similar way as the soybean or broad bean enzyme did. Bean and Hassid19) showed that magnesium chloride (10^{-3} M) stimulated sucrose synthesis in their partially purified pea enzyme, but they attributed the

---

19) R. C. Bean and W. Z. Hassid, *J. Am. Chem. Soc.*, **77**, 5737 (1955).

magnesium effect to the formation of F–6–P from fructose leading to the formation of sucrose phosphate according to reaction (2) which did not take place in the absence of this ion. Turner[10] gave evidence that magnesium was essential in the formation of sucrose from G–1–P and fructose in the presence of a nucleotide fraction from yeasts by the action of a partially purified enzyme preparation from peas. Because sucrose synthesis in that system may involve a complicated sequence of reactions, it is not certain whether magnesium is essential in the reaction (1) which may represent most probably the final step in that reaction sequence. The results given in this paper show that the enzyme solution used in these experiments is active in the absence of added magnesium. However, the increased activity in its presence as well as partial inhibition by Versene and fluoride and its reversal by magnesium indicates that magnesium is requisite for this enzyme. A more direct evidence for the essential role of magnesium must await further purification of the enzyme.

The results given in Fig. 6 show that under the conditions of Fig. 5 fructose may be liberated from F–6–P by the action of contaminating phosphatase in an amount sufficient to account for the synthesis of the sucrose-like substance in the reaction of UDPG with F–6–P. However, the possibility that the enzyme preparation may be contaminated by an enzyme catalyzing reaction (2) is not excluded. Further purification of the enzyme is necessary before a unequivocal answer is given to this problem.

Although the stoichiometry and the reversibility of reaction (1) was substantiated by Leloir et al.[5,6], they could not obtain a true equilibrium of this reaction, because the most purified wheat germ enzyme they obtained was still contaminated by a small amount of invertase. The value for $K = (\text{sucrose} \times \text{UDP})/(\text{UDPG} \times \text{fructose})$ obtained by them varied from 2 to 8 at 37° and pH 7.4. As shown in Table V, the soybean enzyme used in the author's experiments also contained a small amount of invertase contaminant. When corrections are made for the possible interference by invertase, the results shown in Figs. 1 and 7 give 13.7 and 11.4, respectively, as the value of K. Employing the mean value of 12.6, the ΔF° at 37° and pH 7.4 is calculated to be about 1560 cal.

TABLE V. HYDROLYSIS OF SUCROSE BY THE SOYBEAN ENZYME

The reaction mixture contained 100 μmoles of tris-maleate buffer of pH 7.4, 4.4 μmoles of sucrose and 0.1 ml of soybean enzyme; total volume, 0.75 ml. Somogyi's colorimetric method[20] was used for the determination of reducing sugar, which was calculated as glucose.

	Time in hours				
	0.5	1	2	5	25
μmoles of glucose formed	0	0.01	0.02	0.07	0.21
percentage hydrolysis	0	0.22	0.45	1.47	4.63

UDP formed from UDPG as a consequence of sucrose synthesis must be regenerated in some way if sucrose synthesis occurs continuously in the plant tissues. Enzymes which can perform this resynthesis of UDPG by the following reactions have been known in yeasts and in animal tissues[21,22]:

$$\text{UDP}+\text{ATP} \rightleftharpoons \text{UTP}+\text{ADP} \tag{3}$$

$$\text{UTP}+\text{G-1-P} \rightleftharpoons \text{UDPG}+\text{PP} \tag{4}$$

$$\text{PP}+\text{H}_2\text{O} \longrightarrow 2\text{P}i \tag{5}$$

$$\text{Sum: } \text{UDP}+\text{ATP}+\text{G-1-P}+\text{H}_2\text{O} \rightarrow \text{UDPG}+\text{ADP}+2\text{P}i$$

The evidence presented in Fig. 7 indicates that the enzymes catalyzing reactions (3) and (4) are also operative in plants. However, the enzyme solution used in these experiments might not contain pyrophosphatase catalyzing reaction (5), because more sucrose would be formed in its presence than that seen in the figure. It is interesting to note that, although the mitochondrial fraction obtained from broad beans contained the enzyme for sucrose synthesis according to reaction (1), it is devoid of the ability to regenerate UDPG. Either one of the enzymes catalyzing reactions (3) and (4) or both might be absent in this fraction. As the nucleotide diphosphokinase, catalyzing reaction (3), is reportedly present in the rat liver mitochondrial fraction[23], it will be an interesting problem to study the localization of these enzymes in plant cells.

---

[20] M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).

[21] A. Kornberg, *Advances in Enzymol.*, **18**, 191 (1957).

[22] O. Hoffmann-Ostenhof and L. Šlechta, Proc. Internatl. Symp. on Enzyme Chem., p. 180. Maruzen (Tokyo, 1958).

After completion of this work[24], Burma and Mortimer[25] reported that in the homogenates of sugar beet leaves UDPG was synthesized from UTP and G-1-P; UDP plus ATP could replace UTP. According to them, in this plant material sucrose was synthesized from UDPG and F-6-P according to reaction (2) both in vivo and in vitro; fructose could not replace F-6-P. The existence of uridyl transferase (UDPG pyrophosphorylase), catalyzing reaction (4), was substantiated by Neufeld et al.[26] and Turner and Turner[27] in extracts from mung bean seedlings, pea seeds and from a number of other plants.

Acknowledgment The author wishes to express his sincere thanks to Prof. S. Funahashi for his interest in this work.

---

[23] E. Herbert, V. R. Potter and Y. Takagi, *J. Biol. Chem.*, **213**, 923 (1955).

[24] M. Nakamura, Abstracts of Papers, Natl. Meeting of Agr. Chem. Soc. Japan, p. 20 (1956).

[25] D. P. Burma and D. C. Mortimer, *Arch. Biochem. Biophys.*, **62**, 16 (1956).

[26] E. F. Neufeld, V. Ginsburg, E. W. Putman, D. Fanshier and W. Z. Hassid, *Arch. Biochem. Biophys.*, **69**, 602 (1957).

[27] D.H. Turner and J. F. Turner, *Biochem. J.* **69**, 448 (1958).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 405~407, 1959]

Biochemical Studies on "Bakanae" Fungus. Part 48

A New Gibberellin, Gibberellin A_4

By Nobutaka TAKAHASHI, Yasuo SETA, Hiroshi KITAMURA and Yusuke SUMIKI

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo

Received December 17, 1958

The outline of this report[1] has already been published in this Journal appearing under "Communication to the Editors". A new gibberellin which was named gibberellin A_4 was isolated and characterized.

In this report, we wish to report on the isolation and characterization of a new gibberellin which was named gibberellin A_4.

The crude crystals of methyl esters of gibberellins obtained from the broth filtrate of the tank culture using the medium shown in Table I were subjected to chromatography of alumina. As the eluting solvent, ethyl acetate-benzene (1 : 5 by volume) was used. From the fraction eluted earlier than other fractions, containing all known gibberellins, a new crystal, m.p. 175~6°, was obtained in a relatively low yield which was different from the methyl esters of gibberellins A_1, A_2 and A_3 (gibberellic acid). As all the methyl esters of these gibberellins did not show the physiological activity, it was desireble to isolate the free acid of this new methyl ester in order to determine whether this new crystal has physiological activity or not. Therefore, the partition chromatography of a mixture of free gibberellins was attempted by application of the following procedure. Silicic acid treated with pH 5.4 phosphate buffer and solvent of ethyl acetate-benzene (1 : 1 by volume) was used. The first fraction gave a crystal which showed d.p. 220° after recrystallization. The methyl ester of this free acid was identical with the new methyl ester obtained from chromatography of alumina. As shown in Table II, this new acid showed the characteristic physiological activity of about the same degree as gibberellin A_1 by our testing method using rice seedlings but inhibited the growth of root evidently. As this substance is the fourth gibberellin obtained from culture media of Gibberella fujikuroi, we named it gibberellin A_4. At first, there was some incoincidence between analytical data of free acid and methyl ester. However, from the analytical datum of the most purified gibberellin A_4 methyl ester which was obtained from the rechromatography of alumina using ethyl acetate-benzene (1 : 10 by vol.) and analysis of other derivatives, $C_{20}H_{26}O_5$ was assigned to the methyl ester and $C_{19}H_{24}O_5$ to the free acid. By treating the methyl ester with pyridine and acetic anhydride at room temperature for 48 hours, monoacetly gibberellin A_4 monomethyl ester, $C_{22}H_{28}O_6$, m.p. 131~2° was obtained. As the infrared spectrum of this monoacetyl derivative shows no hydroxyl absorption band, it is clear that it has one hydroxyl group. When methyl ester was subjected to the hydrogenation in methanol by Adams' catalyst, one mole of hydrogen was uptaken, resulting dihydrogibberellin A_4 methyl ester, $C_{20}H_{28}O_6$ m.p. 141°. Ozonolysis of gibberellin A_4 methyl ester gave formaldehyde which was identified as formdimedon and a neutral substance $C_{19}H_{22}O_6$, d.p. 201~3°. This result shows the existence of one exocyclic methylene in gibberellin A_4.

---

[1] N. Takahashi, Y. Seta, H. Kitamura and Y. Sumiki, This Bulletin, **21**, 396 (1957).

TABLE I. THE COMPOSITION OF CULTURE MEDIUM

glycerol	3%	$MgSO_4 \cdot 7H_2O$	0.5%
NH_4Cl	0.5%	$ZnSO_4 \cdot 7H_2O$	0.004%
KH_2PO_4	0.5%	$FeSO_4 \cdot 7H_2O$	0.004%
tartaric acid	0.3%	pH 4.8	

TABLE II. ABNORMAL ELONGATION GROWTH TEST USING RICE SEEDLINGS FOR SEVEN DAYS

(Figures in Table indicated length of stems, mm)

Concentration of Gibberellin %	Gibberellin A_1	Gibberellin A_4
0.0016	164	193
0.0005	164	159
0.00025	156	116
0.0001	139	93
control (dist. water)	95	

Infrared spectrum of gibberellin A_4 methyl ester shows a carbonyl band at 1770 cm^{-1} which is characteristic to γ or a somewhat strained δ-lactone and ester carbonyl at 1718 $cm.^{-1}$ Titration of acid shows that this acid is monobasic and analysis of OCH_3 of methyl ester coincides to the above result. Dehydrogenation by Se gave a mixture of 1-methyl and 1,7-dimethyl fluorene. From the above data, it is concluded that gibberellin A_4 has one hydroxyl, one carboxyl, one lactone and one exocyclic methylene and a perhydrofluorene as the carbon skeleton.

EXPERIMENTAL

1) Isolation of gibberellin A_4 methyl ester

A portion of 600 mg of gibberellin mixture was dissolved in methanol and treated with etherial diazomethane. The residual matter obtained from evaporation of the solvent was dissolved in 15 ml of ethyl acetate-benzene (1 : 5 by volume). A column was prepared from 30 g of alumina and the same composition of solvent. A solution containing the sample was eluted from the top of the column. Elution was continued with the same solvent composition. After about 200 ml of solvent was eluted, a crystal appeared in the following effluent on evaporation. After the quantity of the crystal decreased, gibberellin A_1 again appeared in the effluent. The collected first fraction (50 mg) was rechromatographed in the same manner using the same solvent (1 : 10 by vol.). It gave the most purified gibberellin A_4 methyl ester. Recrystallization from ethyl acetate and ligroin gave a crystal of m.p. 175~6°. $[\alpha]_D^{15}-14.2$ (c=3.17, CH_3OH)

Found: C, 69.01; H, 7.28; OCH_3, 8.09. Calcd. for $C_{20}H_{26}O_5$: C, 69.34; H, 7.57; OCH_3, 8.52.

2) Isolation of free gibberellin A_4

A portion of 440 mg of gibberellin mixture was dissolved in 40 ml of ethyl acetate and 40 ml of chloroform was added to this solution. A column was prepared by packing 60 g of silicic acid treated with 40 ml of pH 5.2 phosphate buffer, using a mixture of ethyl acetate-benzene (1 : 1 by vol.). The solution of the sample was added to this column and immediately eluted. Every 20 ml of effluent was collected as one fraction. From fractions 3~9, 20 mg of a new crystal was obtained and from fractions 12~26, 330mg of a mixture of gibberellins A_1 and A_3 was obtained. The first fraction was recrystallized from ethyl acetate-ligroin into needles, d.p. 222°. $[\alpha]_D^{20} -14.70$ (c=3.81, CH_3OH)

Found: C, 67.55; H, 7.35; Molecular weight, 343 (Rest), 334 (titration). Calcd. for $C_{19}H_{24}O_5$: C, 68.65; H, 7.28; molecular weight 332.

3) Catalytic hydrogenation of gibberellin A_4 methyl ester

Gibberellin A_4 methyl ester (200 mg) was hydrogenated in methanol with Adams' catalyst. One mole of hydrogen was uptaken in 15 minutes. One hundred and eighty mg of residual matter obtained from evaporation of solvent was crystallized from ethyl acetate-ligroin into rods, m.p. 141°.

Found: C, 69.01; H, 7.28. Calcd. for $C_{20}H_{28}O_5$: C, 69.34; H, 7.57.

4) Acetylation of gibberellin A_4 methyl ester

A portion of 200 mg of gibberellin A_4 methyl ester was dissolved in 10 ml of pyridine and 5 ml of acetic anhydride and left at room temperature for 48 hours. Solvent was distilled off in vacuo. The residual matter was treated with acidic water and extracted with ethyl acetate. Ethyl acetate was washed with aq. $NaHCO_3$. On evaporation and recrystallization from ligroin, a crystal of m.p. 131~3° (150 mg) was obtained.

Found: C, 68.17; H, 7.29. Calcd. for $C_{22}H_{28}O_6$: C, 68.02; H, 7.27.

The number of acetyl group was one, as determined by the Kuhn-Roth method.

5) Ozonolysis of gibberellin A_4 methyl ester

A portion of 200 mg of gibberellin A_4 methyl ester was dissolved in 70 ml of ethyl acetate. Ozone was passed through this sample solution which was cooled by dry ice and alcohol mixture. At room temperature, the solvent was evaporated in vacuo and the residual matter was steam distilled. An amount of 200 ml of distillate was collected and it was treated with dimedon alcohol solution. Immediately, fine needles precipitated. Recrystallization gave a crystal of m.p. 186~9° which was identical with formdimedon in all respects. The non-volatile fraction was extracted with ethyl acetate. The neutral fraction (140 mg) was chromatographed on alumina using solvent of ethyl acetate-benzene (5 : 1 by vol.). From the first-60 ml of the effluent, 100 mg of crystals was obtained. Recrystallization of this crystal gave rods, m.p. 203°.

Found: C, 65.25; H, 7.37. Calcd. for $C_{19}H_{24}O_6$: C, 65.50; H, 6.94.

6) Dehydrogenation by selenium

A mixture of gibberellin A_4 methyl ester (300 mg) and selenium powder (1.3 g) was heated in a slow stream of nitrogen at 350~60° for 2 hrs. The residual solid was extracted with ether and then washed with aq. $NaHCO_3$. On evaporation, 80 mg of an oily product was obtained. The oily product was distilled at 140~60°/2 mm Hg. The colourless syrup thus obtained was chromatographed on alumina (10 g) with light petroleum ether. Each 8 ml of the effluent was collected as one fraction. Fractions 4~6 gave a crystal which was recrystallized into rods, m.p. 80~92°. Infrared and ultraviolet spectra of this crystal was very similar to that of a mixture of 1-methyl and 1, 7-dimethyl fluorene.

Acknowledgment We are indebted to Mr. Aizawa for the infrared spectra and to Mrs. Sato, Miss Suzuki and Miss Isobe for micro analysis. The authors wish to express their thanks to Kyowa Fermentation Co. for supplying the gibberellins. This research has been supported in part by funds from the Ministry of Education.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 408~411, 1959]

Biochemical Studies on "Bakanae" Fungus. Part 49

Chemical Structure of Gibberellins. Part XIV

By Hiroshi KITAMURA, Yasuo SETA, Nobutaka TAKAHASHI,
Akira KAWARADA and Yusuke SUMIKI

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo

Received December 17, 1958

The outline of this report has already been published in this Journal appearing as "Communication to the Editors". The molecular formula of gibberellin A_2 has been corrected to $C_{19}H_{26}O_6$. To elucidate the relation between the four gibberellins, we studied their functional groups, skeleton and double bond. As for the results, it was certified that gibberellin A_1, A_2, A_3 (gibberellic acid) and A_4 have functional groups and skeleton as shown in Table I.

We isolated and characterized four biological active compounds, i.e. gibberellins A_1, A_2, A_3 (gibberellic acid and Stodola's gibberellin X) and A_4 as previously reported.[1,2] Concerning gibberellin A_2 (G A_2), we have corrected its molecular formula and that of its methyl ester to $C_{19}H_{26}O_6$ and $C_{20}H_{28}O_6 \cdot H_2O$ respectively, from the results of the determination of the molecular weight by the X-ray method (lattice constant- a: 9.66, b: 10.69, c: 18.96 Å; specific density: 1.30; Mol. Wt. 385; theor. 382) and of the crystalline water (found. 4.93; theor. 4.71%) of G A_2 methyl ester by the method of Karl Fisher and from the following analytical data of its derivatives and degradation products.

We have elucidated the functional groups and skeleton of G A_1 and A_2 from the following experimental results tnd those of four gibberellines are summarized in Table I.

Both G A_1 and G A_2 form the corresponding neutral monomethyl esters by treatment with diazomethane and show the infrared bands (in Nujol) at 1763, 1755 cm^{-1} (γ or δ-lactone) respectively, showing that they contain one carboxylic and one lactone group. G A_1 methyl ester absorbs one mole of hydrogen in catalytic hydrogenation, resulting two dihydro G A_1 methyl esters, which are able to separate each other only through chromatography on alumina after acetylation (see below), but G A_2 methyl ester absorbs no hydrogen, showing that G A_1 has one C=C double bond and G A_2 no double bond. G A_1 methyl ester gives one monoacetyl derivative, $C_{22}H_{28}O_7$, m.p. 173~4°, by treatment with acetic anhydride and pyridine at room temperature and gives a syrup by boiling with acetic anhydride and anhydrous sodium acetate. Hydrogenation of this syrup affords two diacetyl derivatives of dihydro G A_1 (see below).

Dihydro G A_1 methyl ester in pyridine and acetic anhydride at room temperature for 48 hrs. gives two monoacetyl derivatives, $C_{22}H_{30}O_7$, m.p. 120~2°, 202~4°, and two diacetyl derivatives, $C_{24}H_{32}O_8$, m.p. 176~8°, 210~2°, separated by chromatography on alumina. These acetyl derivatives, which are quite similar but not identical in infrared spectra, are thought to be stereoisomers, each other, which were derived from one asymmetric carbon created as the result of hydrogenation.

Dihydro G A_1 is oxidized to a monoketone compound $C_{19}H_{24}O_6$, d.p. 256~8°, with chromic acid and shows high stability against further

---

[1] N. Takahashi, H. Kitamura, A. Kawarada, Y. Seta, M. Takai, S. Tamura and Y. Sumiki, This Bulletin, **19**, 267 (1955).

[2] N. Takahashi, Y. Seta, H. Kitamura and Y. Sumiki, in this preceding paper.

TABLE I

Gibberellins	Mol. formula	M.P.$_{(0)}$	$[\alpha]_{D(0)}$	-OH		-COOH	-CO-O-	C=C	Skeleton
				sec.	tert.				
A_1	$C_{19}H_{24}O_6$	232−5	+42.3	1	1	1	1	1	1,7-dimethyl-fluorene
A_2	$C_{19}O_{26}O_6$	235−7	+11.7	1	1	1	1	0	
A_3	$C_{19}H_{22}O_6$	233−5	+86.0	1	1	1	1	2	
A_4	$C_{19}H_{24}O_5$	222	−14.7	1	0	1	1	1	

oxidation, showing that one is secondary and the other is tertiary among the two hydroxyl groups. G. A_2 methyl ester also gives a monoacetyl derivative, $C_{20}H_{30}O_7$, m.p. 204~5°, by treatment with acetic anhydride and pyridine at room temperature, and is oxidized to a monoketone compound, $C_{20}H_{26}O_6 \cdot H_2O$, m.p. 102~4°, with chromic acid. The presence of the ketone group is confirmed from the monoketoxime, $C_{20}H_{27}O_6N$, m.p. 206~8°. Both monoacetyl and monoketone derivatives of G A_2 methyl ester still show an OH band (3510 cm^{-1}) in infrared spectra.

From the above results, it was elucidated that both G A_1 and G A_2 are dihydroxy (one is secondary and the other tertiary) lactonic monocarboxylic acids, and the former has one ethylenic double bond while the latter does not possess an ethylenic double bond.

The selenium dehydrogenation of G A_1 methyl ester at 320~30°, gives 1,7-dimethyl-fluorene, $C_{14}H_{15}$, m.p. 106~7°, as the only one crystalline

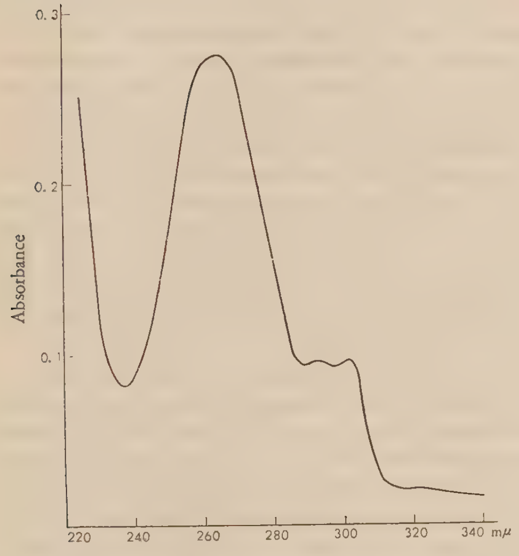


FIG. 1. Ultraviolet Spectrum of Gibbaren

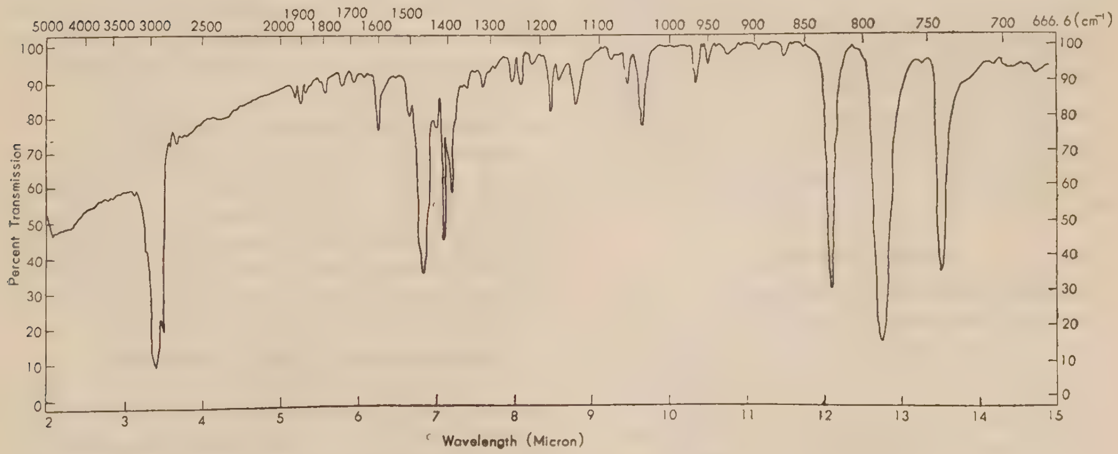


FIG. 2. Infrared Spectrum of Gibberen

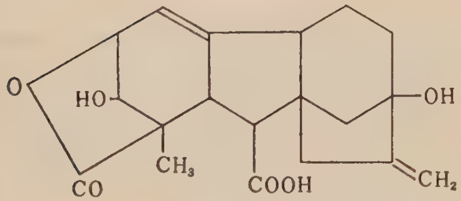


FIG. 3.

product which was identified by mixed m.p. and comparison of its ultraviolet absorption spectrum over the range of 220~320 mμ, and infrared spectrum with those of the synthesized specimen and by comparison of their trinitro benzoates and picrates. (Figs. 1, 2)

When the dehydrogenation of G A$_2$ methyl ester is carried out at 350~60°, 1,7-dimethylfluorene and a small amount of a neutral oily substance are isolated, and the ultraviolet absorption spectrum of the latter is quite similar to the former.

Cross et al.[3,4] reported that G acid, $C_{19}H_{22}O_6$, is shown to be a tetracyclic dihydroxy (one is secondary and the other tertiary) lactonic acid with two ethylenic double bonds, having a perhydrofluorene skeleton, as shown in Fig. 3.

From the facts described above, it was determined and summarized in Table I that G A$_1$, A$_2$ and A$_3$ have the same functional groups and skeleton, except the numbers of the carbon double bond (A$_1$: 1, A$_2$: 0, A$_3$: 2) and A$_4$, $C_{19}H_{24}O_5$, has been shown to be a tetracyclic monohydroxy (secondary) lactonic acid with one ethylenic double bond having the same skeleton as the other gibberellins, as described in this preceding paper[2]. The relation between the chemical structure of four gibberellins will be described in the following paper.

EXPERIMENTAL

1) Acetylation of G A$_1$ methyl ester

a) G A$_1$ methyl ester (120 mg) in pure dry pyridine (5 cc) and acetic anhydride (3 cc) was left at room temperature for 48 hrs. Extraction, chromatography on alumina (Brockmann) and elution with benzene gave, first, a diacetyl derivative (40 mg), m.p. 168~9° (from ethyl acetate-ligroin), *Anal.* Found: C, 64.74; H, 6.70. Calcd. for $C_{24}H_{30}O_8$: C, 64.6; H, 6.70, and after elution with ethyl acetate-benzene (1 : 10) gave a monoacetyl derivative (30 mg), m.p. 173~4° (from ethyl acetate-ligroin), *Anal.* Found C, 65.58; H, 6.79. Calcd. for $C_{22}H_{28}O_7$: C, 65.3; H, 6.9.

b) G A$_1$ methyl ester (200 mg) in acetic anhydride (5 cc) and anhydrous sodium acetate (0.5 g) was heated under reflux for 1.5 hr. Chromatography on alumina and elution with benzene gave only a syrup (180 mg). The syrup in methanol (10 cc) was hydrogenated over palladised charcoal (5%, 50 mg) (uptake 1 mole). Chromatography of the product on alumina afforded on elution with ethyl acetate-benzene (1 : 99), dihydro G A$_1$ diacetyl derivative (70 mg), m.p. 176~8°. *Anal.* Found: C, 64.21; H, 7.15. Calcd. for $C_{24}H_{32}O_8$: C, 64.27; H, 7.19; and then elution with ethyl acetate-benzene (1.5 : 98.5) another dihydro G A$_1$ diacetyl derivative, (70 mg), m.p. 210~2°. *Anal.* Found: C, 64.21; H, 6.93.

2) Acetylation of dihydro G A$_1$ methyl ester

Dihydro G A$_1$ methyl ester (300 mg), in pure pyridine (15 cc) and acetic anhydride (8 cc) was left at room temperature for 48 hrs. Chromatography on alumina and elution with ethyl acetate-benzene (the ratio of the solvent, first 1 : 99 and then 1.5 : 98.5) gave two dihydro G A$_1$ diacetyl derivatives m.p. 176~8°, and 210~2° (30 mg), respectively, and then elution with ethyl acetate-benzene (3 : 97) at first, gave, dihydro G A$_1$ monoacetyl derivative (60 mg) m.p. 120~2°. *Anal.* Found: C, 64.55; H, 7.54. Calcd. for $C_{22}H_{30}O_7$: C, 65.01; H, 7.44. Then, further elution with the same solvent furnished another dihydro G A$_1$ monoacetyl derivative (90 mg), m.p. 202~4°. *Anal.* Found: C, 64.99; H, 7.51.

3) Chromic acid oxidation of dihydro G A$_1$

Dihydro G A$_1$ (150 mg) in acetic acid (5 cc) was treated with 1.2% chromic acid-acetic acid solution (5 cc) at 60° for 3 hrs. (consumption of 1 equiv. of oxygen). Isolation of the product in the usual way afforded a monoketone derivative (80 mg), m.p. 256~8° (from alcohol-ligroin). *Anal.* Found: C, 65.22; H, 7.17. Calcd. for $C_{19}H_{24}O_6$; C, 65.5; H, 6.9.

4) Dehydrogenation of G A$_1$ methyl ester

A mixture of G A$_1$ methyl ester (400 mg) and selenium powder (800 mg) in a small distillation flask was heated to 320~30° for 2 hrs. The residue was extracted with ether and washed with dilute sodium bicarbonate. The

---

[3] B. E. Cross, J. F. Grove, J. MacMillan and T.D.C. Mulholland, *Chem. and Ind.*, **36**, 954 (1956).

[4] B. E. Cross, *Journ. of Chemical Society*, **1954**, 4670.

recovered neutral fraction (260 mg) was distilled at ca. 1 mm, yielding an oily product (140 mg). Chromatography of this product on alumina (16×1.2 cm) on elution with light petroleum (b.p. 35~60°) afforded 1,7-dimethylfluorene (gibberen) (20 mg) as plate, m.p. 106~7° (from methanol), identical with the authentic specimen in respects of m.p., ultraviolet and infrared spectra.

5) Acetylation of G A_2 methyl ester

G A_2 methyl ester (150 mg) in pure dry pyridine (10 cc) and acetic anhydride (50 cc) was left at room temperature for 48 hrs. Chromatography on alumina and elution with ethyl acetate-benzene (3 : 97) gave a monoacetyl derivative (140 mg), m.p. 204~5° (from ethyl acetate-ligroin) $[\alpha]_D^{10}+40.0$ (c, 2.5 in ethanol). *Anal.* Found: C, 65.08; H, 7.32. Calcd. for $C_{22}H_{30}O_7$ C, 65.01; H, 7.44. Acetylation of G A_2 methyl ester with the above reagents at room temperature for 7 days, also gave the same monoacetyl derivative, m.p. 204~5°.

6) Chromic acid oxidation of G A_2 methyl ester

G A_2 methyl ester (150 mg) in acetic acid (5 cc) was treated with 1.2% chromic acid-acetic acid solution (5 cc) at 60° for 3 hrs. Isolation of the product in the usual way and chromatography on alumina afforded, on elution with ethyl acetate-benzene (1 : 10) a monoketone derivative (30 mg), m.p. 102~4°, anhydrous m.p. 177~8°. *Anal.* Found: C, 63.23; H, 8.02. Calcd. for $C_{20}H_{26}O_6 \cdot H_2O$. C, 63.14; H, 7.42; Anhydrous, *Anal.* Found: C, 65.98; H, 6.43, Calcd. for $C_{20}H_{26}O_6$ C, 66.3; H, 7.2; λ_{max} 290 mμ $\varepsilon=50$. The oxidation product (24 mg) was boiled with hydroxylamine hydrochloride (25 mg), anhydrous sodium acetate (50 mg), alcohol (3 cc) for 5 hrs. Isolation of the residue in the usual way gave a monoketoxime, m.p. 206~8° (from alcohol). *Anal.* Found: C, 63.73; H, 6.73; N, 3.81. Calcd. for $C_{20}H_{27}O_6N$ C, 63.64; H, 7.21; N, 3.71.

7) Dehydrogenation of G A_2 methyl ester

A mixture of G A_2 methyl ester (500 mg) and selenium powder (1.7 g) was heated in a slow stream of nitrogen to 320° in 30 min. (evolution of carbon dioxide), then raised to and kept at 350~60° for 2.5 hrs. No volatile carbonyl compound was detected (2,4-dinitrophenylhydrazine trap). The residue was extracted with ether and then washed with sodium hydrogen carbonate solution, yielding an oily product (120 mg). The oily product was distilled at 140~160° ca. 2 mm. Chromatography on alumina (20×1.5 cm) on elution with light petroleum (b.p. 35~60°), afforded an oily substance and by further elution, furnished 1,7-dimethylfluorene (50 mg), m.p. 100~103°, identical with ultraviolet and infrared spectra. *Anal.* Found: C, 92.47; H, 7.04. Calcd. for $C_{15}H_{14}$ C, 92.7; H, 7.3. The picrate formed orange-red needles, m.p. 83~4°. The 1,3,5-trinitrobenzene adduct crystallized from ethanol in yellow needles, m.p. 98~100°, was not depressed by admixture with an authentic specimen.

Acknowledgements The authors wish to thank Prof. Y. Go, Faculty of Textile, the University of Shinshu, for the X-ray measurement, the Central Research Institute of Japan Monopoly Corp., Mr. K. Aizawa for the infrared measurements, and the Laboratory of Microanalysis, Department of Agricultural Chemisiry, University of Tokyo, for the microanalysis. We are also indebted to Kyowa Fermentation Industry Co., Ltd., Takeda Pharmaceutical Co., Ltd., and Meiji Nyūgyo Co., Ltd. for kindly supplying gibberellins used in this work. This study was supported in part by the Grant in Aid for Agricultural Research from the Ministry of Education, to which the authors express their thanks.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 412~417, 1959]

Biochemical Studies on "Bakanae" Fungus. Part 50

Chemical Structure of Gibberellins. Part XV

By Yasuo SETA, Nobutaka TAKAHASHI, Akira KAWARADA,
Hiroshi KITAMURA and Yusuke SUMIKI

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo

Received December 17, 1958

The outline of this report has already been published in this Journal as a short communication[1] to the Editors. The presence of the $-\overset{|}{C}(OH)-\overset{|}{C}=CH_2$ group and a five-membered ring in gibberellin A_1 was elucidated by ozonolysis. The points of attachment of this five-membered ring and the position of the tertiary hydroxyl group were also elucidated by dehydrogenation of the ozonolysis product.

As it has been described in the previous paper[2], gibberellin A_1 (G A_1) (I) certainly has the perhydrated fluorene as its mother nucleus, and has one carboxyl, one lactone, two hydroxyls, probably one secondary and another tertiary, and one ethylenic double bond as functional groups. From these facts and the molecular formula, the tetracyclic nature of G A_1 had been suggested.

This paper deals with the nature of the ethylenic double bond and its relative position towards tertiary hydroxyl and also the ring system.

The ethylenic double bond in G A_1 exists as the form of terminal methylene. This is suggested from the spectroscopic evidence and is confirmed by ozonolysis. The G A_1 methyl ester shows an infrared absorption band 890 cm^{-1} and this band disappears by catalytic hydrogenation. On the ozonolysis of G A_1 methyl ester, the following four products: formaldehyde (ca. 45% of the theoretical amount), formic acid (ca. 33%), a neutral compound $C_{19}H_{24}O_7$ (II) and an acid $C_{19}H_{24}O_8$ (III), are obtained. (II) shows infrared absorption at 1765 cm^{-1} (lactone), 1735 cm^{-1} (ester carbonyl) and 1750 cm^{-1}, suggesting the presence of 5-membered ring ketone, and is oxidised to (III) by hydrogen peroxide in alkaline solution. It gives negative Schiff's reaction but reduces ammoniacal silver nitrate solution or Fehling's solution, suggesting it to be α- or β-ketol. It is not affected by periodic acid, but its reduction product with sodium borohydride readily consumes periodic acid, producing an aldehydic substance. From these facts, (II) is considered to be α-ketol.

The acidic product (III) shows infrared absorption at 1758 cm^{-1} (lactone), 1732 cm^{-1} (ester carbonyl), 1703 cm^{-1} (carboxyl) and its methyl ester (IV) shows a weak ultraviolet absorption at 278 mμ and infrared absorption at 1763 cm^{-1} (lactone), 1730cm^{-1} (ester carbonyl) and 1713cm^{-1}, suggesting the presence of 6-membered ring ketone. When (IV) was acetylated with acetic anhydride and pyridine or acetic anhydride and anhydrous sodium acetate, only a monoacetate (V) is obtained and O–H stretching absorption near 3500 cm^{-1} disappears completely.

Since G A_1 has two hydroxyls, as it had previously been described, this fact shows that one of the hydroxyls of G A_1, perhaps a tertiary one, would undergo some change during ozonolysis. This acetate (V), when oxidised

---

[1] Y. Seta, H. Kitamura, N. Takahashi and Y. Sumiki, This Bulletin, **21**, 73 (1957).

[2] H. Kitamura, Y. Seta, N. Takahashi, A. Kawarada and Y. Sumiki, This Bulletin, **21**, 71 (1957).

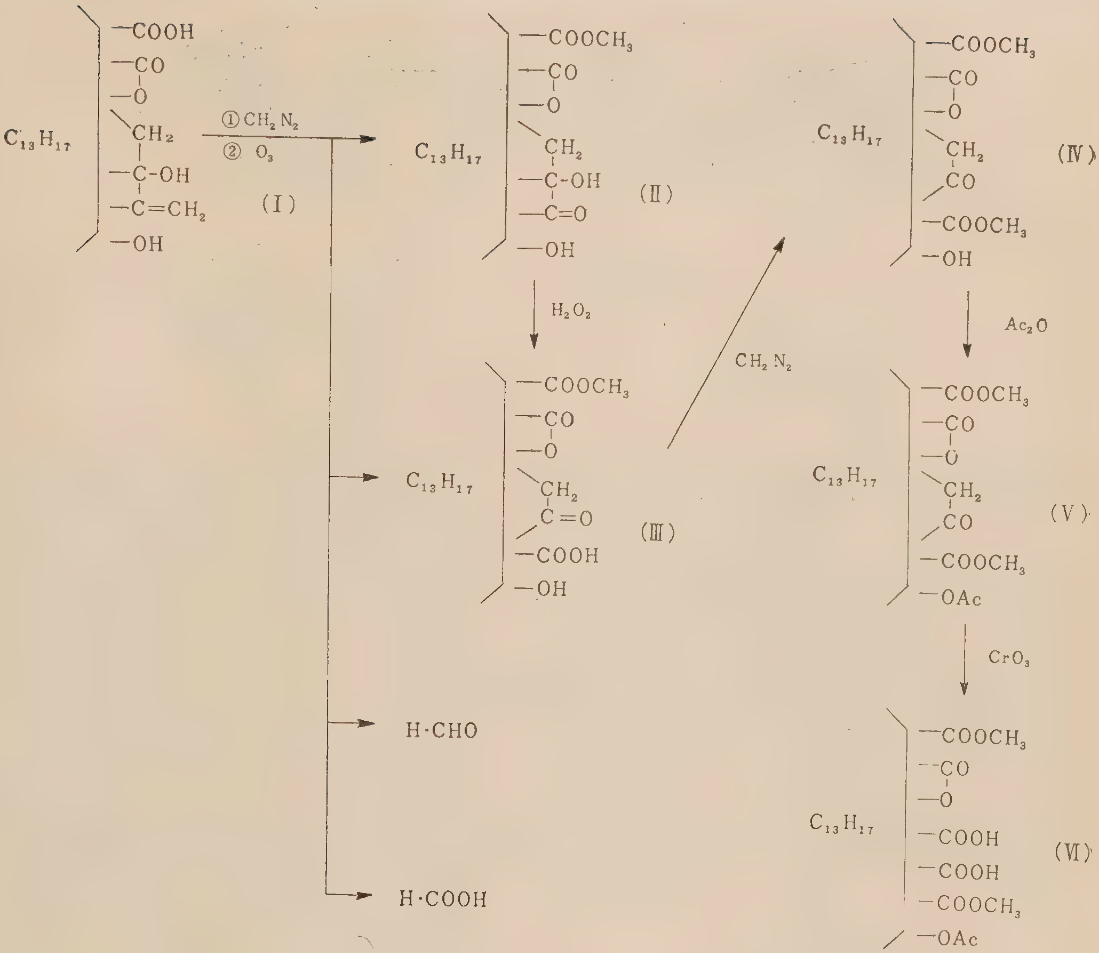


FIG. 1.

with chromic acid in acetic acid solution at 95～100°, gives a dicarboxylic acid (VI) without loss of the carbon atom. Thus, the cyclic nature of its ketone is confirmed chemically.

These reactions described above, are reasonably interpreted by the following partial structure given for G A_1 (Fig. 1).

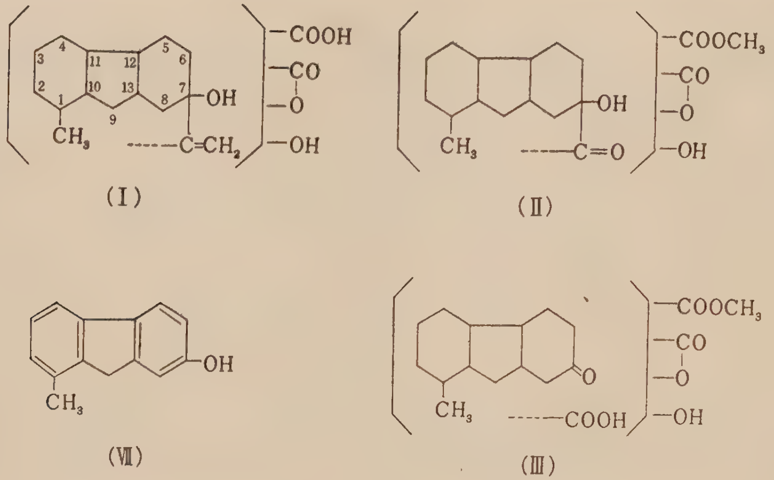
The ketone of (III) is elucidated to exist in the perhydrated fluorene nucleus by the following experiment. When (III) was dehydrogenated with selenium under the same reaction condition as in the case of obtaining 1,7-dimethyl fluorene[3)] from G A_1 methyl ester, a hydroxyl compound, $C_{14}H_{12}O$, m.p. 159～161°, was obtained, which was identified as 1-methyl-7-hydroxyl fluorene (VII) by comparison with a synthetic specimen, which was kindly sent from Dr. J. F. Grove of Imperial Chemical Industries Ltd., England. The fact that the 6-membered ring ketone remains in the form of phenol in the case of selenium dehydrogenation under a relatively mild reaction condition, had been pointed out by Ruzicka[4)], Peak and Robinson[5)], etc.. Therefore, the ketone of (III) should be located at the 7-position of

---

3) H. Kitamura, Y. Seta, N. Takahashi, A. Kawarada and Y. Sumiki, This Bulletin, **21**, 71 (1957).

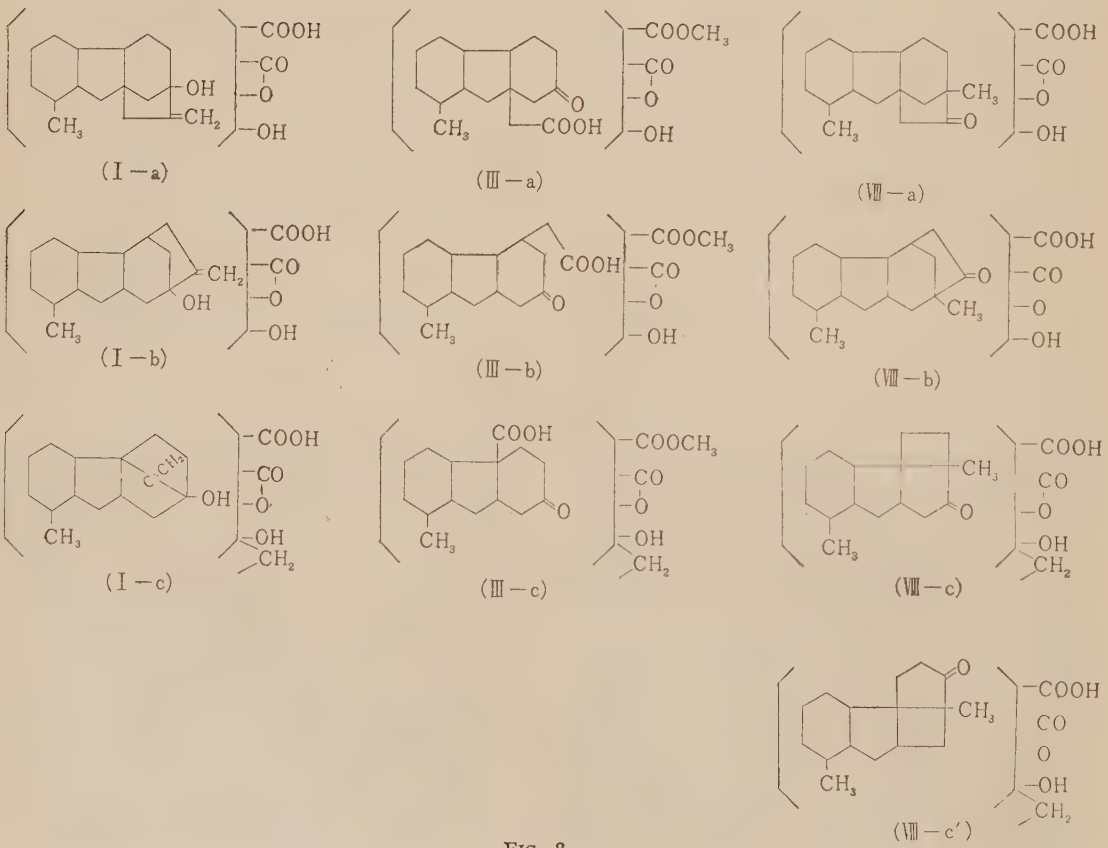
4) L. Ruzicka, *Helv. Chim. Acta*, **19**, 419 (1936).

5) D.A. Peak and R. Robinson, *J. Chem. Soc.*, **1936**, 756.



(I) (II) (VII) (III)

FIG. 2.



(I−a) (III−a) (VIII−a) (I−b) (III−b) (VIII−b) (I−c) (III−c) (VIII−c) (VIII−c′)

FIG. 3.

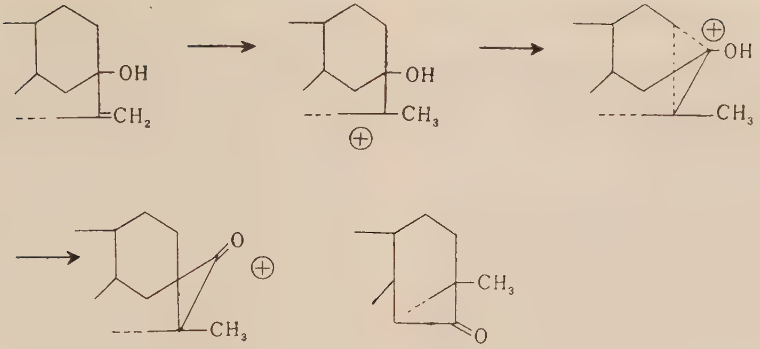


FIG. 4.

the perhydrated fluorene. And further, as this ketone, together with carboxyl group, has been produced by the oxidative cleavage of cyclic α-ketol linkage of (II), it is reasonable to express the relation between (II), (III) and G A_1 as shown in Fig. 2.

Thus, one end of the 4-th ring including exocyclic methylene was determined to be at C-7 of perhydro-fluorene.

Another end of this ring was determined to be C-13 of perhydro fluorene from the following evidence.

The ketone of (II) was shown to be a 5-membered ring one from infrared investigation, which shows an absorption band at 1750 cm^{-1}. Thus, this 4-th ring should be a 5-membered ring. From this point of view and the number of carbon atom of G A_1, the following three possible formulae (I-a), (I-b) and (I-c) might be considered for G A_1 (Fig. 3).

If the formula (I-b) is correct, the ozonolysis product (III) might be formulated as (III-b), and then, the main product of dehydrogenation must not be 1-methyl-7-hydroxyl fluorene, but 1,5-dimethy-7-hydroxyfluorene. Therefore, the possibility for (I-b) should be excluded. The possibility for the formula (I-c) can also be excluded from the mechanism of proton catalysed rearrangement of G A_1. G A_1 gives gibberellin C[6] (VIII-a) by treatment with acid, and the mechanism of this reaction was elucidated to be similar to that of the Wagner-Meerwein rearrangement, as illustrated in Fig. 4[7]*.

When gibberellin C was dehydrogenated with selenium, 1,7-dimethyl fluorene is obtained as main product. If the formula (I-c) is correct, gibberellin C might be formulated as (VIII-c) or (VIII-c′) from this mechanism and the production of fluorene derivative should not be considered by dehydrogenation. As illustrated in Fig. 3 only the formula (I-a) can fulfil these conditions.

Consequently, we propose the following partial structure (I-a) for gibberellin A_1 as the most probable one.

EXPERIMENTAL

Ozonolysis of gibberellin A_1 methyl ester

A sample of 250 mg of chromatographically purified G A_1 methyl ester (m.p. 232~4°) was dissolved in 30 ml ethyl acetate and ozonised at $-40° \sim -50°$ with oxygen stream containing ca. 3% O_3. After evaporation of the solvent in vacuo at room temperature, the residue was decomposed with water, steam-distilled immediately, and the distillate was treated in the usual way to separate into neutral and acidic fractions. The precipitate (92 mg) from the neutral distillate, obtained by the addition of alcoholic dimedone solution, showed m.p. 188~9.5° after recrystallization from ethanol, and was

---

6) A. Kawarada, H. Kitamura, Y. Seta, N. Takahashi, M. Takai, S. Tamura and Y. Sumiki, This Bulletin, **19**, 278 (1955).

7) N. Takahashi, Y. Seta, H. Kitamura, A. Kawarada and Y. Sumiki, This Bulletin, **21**, 75 (1957).

* Detailed research on the mechanism of this reaction will be described in our next report, Part 51. of this series.

identified as formdimedone by mixed m.p. with an authentic sample.

Acidic distillate, which was neutralized with N/10-NaOH (2.1 ml was required) and evaporated to a small quantity, gave a precipitate of the mercurous chloride by the addition of mercuric chloride solution, showing the presence of formic acid.

Non-volatile fraction was separated into neutral and acidic components by ethyl acetate extraction. The neutral component (77 mg) was chromatographed over alumina (Brockmann 1.2×10 cm) and eluted with benzene-ethyl acetate (5 : 1), obtaining compound (II) as fine needles, m.p. 135° (resolidifies and remelts at 170°) after recrystallization from ethyl acetate-ligroin. $[\alpha]_D^{25}$: +44.0 (c, 0.770 in MeOH), (Found. C, 62.48; H, 7.21. Calcd. for $C_{19}H_{24}O_7$: C, 62.6; H, 6.6).

From the acidic component (170 mg) was obtained compound (III) by silicic acid-partition chromatography, using ethyl acetate as the mobile phase and 1 M phosphate buffer solution of pH 5.4 as the stationary phase. Prisms, m.p. 98° after recrystallization from water. $[\alpha]_D^{25}$: +32.1 (c, 2.090 in MeOH) (Found. C, 59.31 ; H, 6.22. Calcd. for $C_{19}H_{24}O_8$: C, 60.0 ; H, 6.3).

Methyl ester of this acid (IV) was obtained by the Fischer-Speier method or by the action of ethereal diazomethane, fine needles, m.p. 169~70° (from ethyl acetate-ligroin) $[\alpha]_D^{25}$+48.2 (c, 0.891 in MeOH) λ_{max} 275 mμ (ε=63.3) (Found. C, 60.96 ; H, 6.71 ; $-OCH_3$, 16.03. Calcd. for $C_{20}H_{26}O_8$: C, 60.9; H, 6.6; 2−OCH_3, 15.17).

Oxidation of ozonolysis product (II) with hydrogen peroxide

To a 50% aq. methanolic solution (15 ml) of compound (II) (50 mg) were added few drops of 10% NaOH aq. and 5 ml of 3% H_2O_2 solution. The mixture was allowed to stand for 48 hrs at room temperature, extracted with ethyl acetate and the reaction products were treated in the usual way to separate into neutral and acidic fractions. The acidic product (50 mg) crystallized by treatment with water, showing m.p. 95~98°, and no depression on admixture with compound (III). On methylation with diazomethane was obtained its methyl ester of m.p. 168~9°, which was proved to be identical with methyl ester (IV) in mixed m.p. and infrared spectra.

Acetylation of methyl ester (IV)

a) The above methyl ester (100 mg), Ac_2O (5 ml) and anhydrous NaOAc (220 mg) were refluxed for 30 min., poured into water, the precipitate was collected (92 mg) and recrystallized from ethyl acetate-ligroin, needles, m.p. 195°, $[\alpha]_D^{25}$+71.0 (c, 0.887 in MeOH) (Found. C, 60.57; H, 6.64. Calcd. for $C_{22}H_{28}O_9$: C, 60.54 ; H, 6.47).

For the determination of the acetyl-number of this product, the Kuhn-Roth micro method was applied, using 1 N-NaOH-MeOH as the hydrolysing agent. 6.496 mg of sample required 1.518 ml of N/100-NaOH, calcd. amt. required 1.489 ml for 1-Ac.

b) The methyl ester (100 mg), acetic anhydride (2 ml) and pyridine (5 ml) were mixed and allowed to stand for 48 hrs.. The reagents were distilled off in vacuo, water was added and precipitates were collected (90 mg) and recrystallized from ethyl acetate-ligroin. m.p. 194~5°.

Oxidation of acetate (V) with chromic acid to dicarboxylic acid (VI).

A 300 mg sample of the acetate (V) was dissolved in glacial acetic acid (pretreated with chromic acid) and added dropwise with a solution of 320 mg of chromic acid in 90% acetic acid under constant stirring, keeping the reaction temperature at 95~100° on the steam bath. Heating and stirring were continued for 1 hr. after completion of the addition of reagent and finally, MeOH was added to destroy an exess of chromic acid. Acetic acid was evaporated in vacuo and the reaction products were extracted with ether. The extract (260 mg) was purified by silicic acid partition chromatography, using 1 M phosphate (buffer solution of pH 5.4 as the stationary phase and benzene-butanol (97 : 3 − 90 : 10) as the mobile phase. Compound (VI) was obtained as prisms of m.p. 194~6° after recrystallization from ethyl acetate-ligroin. $[\alpha]_D^{25}$+87.2 (c, 0.430 in MeOH) (Found. C, 54.20; H, 5.61 ; $-OCH_3$ 13.33. Calcd. for $C_{22}H_{28}O_{12}$: C, 54.53; H, 5.83; 2–OCH_3, 12.8). A 4.599 mg sample required 1.79 ml of N/100–NaOH for neutralization, calcd. amt. (as dicarboxylic acid) is 1.90 ml.

Dehydrogenation of compound (III) with selenium

A portion of 0.24 g of compound (III) was mixed with 0.5 g selenium powder and dehydrogenated for 3 hrs. at 300~320°; The reaction mixture was extracted with ether, the ether was evaporated and the residues were distilled at 180~200° (bath temperature) (1 mm/Hg). The solidified distilates (33 mg) were chromatographed over alumina (Brockmann, 0.5×10 cm) and eluted with petroleum ether (b.p. 40~60°)–ether (10 : 1), obtaining fine needles of m.p. 159~61° (from ligroin). (Found. C, 84.99 ; H, 7.11. Calcd. for $C_{14}H_{12}O$: C, 85.68 ; H, 6.16). This compound showed a mixed m.p. 164~7° with the synthetic 1-methyl-7-hydroxy-fluorene (m.p. 167°) and both were proved to be identical by comparison of their infrared and ultraviolet absorption spectra.

Dehydrogenation of gibberellin C with selenium A mixture of gibberellin C (500 mg) and selenium powder (1 g) was heated in a long-necked reaction tube at 300~305° for 1 hr., and subsequently 315~20° for 2 hrs.. After cooling, the solid was extracted with ether. The neutral fraction (350 mg) was distilled at 150~60°/1 mm Hg and the distillate (200 mg) was chromatographed over alumina (Brockmann, 1 × 15 cm) and eluted with petroleum ether (40 ml), followed by petroleum ether-ether (70 : 1) (100 ml). A portion of 130 ml of the effluent was collected and evaporated, yielding a crystal (40 mg). This crude crystal was re-chromatographed over alumina and eluted with petroleum ether-ether (50 : 1) (300 ml) and every 5 ml of the effluent was collected. Fractions 3~4 gave a crystal of m.p. 106~7° (12 mg) which was identified as 1,7-dimethyl fluorene by mixed m.p., ultraviolet and infrared spectra. From fractions 19~22, a crystal of m.p. 126~7° was obtained. It was confirmed that this crystal was gibberone[8] from its ultraviolet and infrared spectra.

Acknowledgement The authors are indebted to Mr. M. Nagasawa of the Central Research Institute of Japan Monopoly Corporation and Mr. K. Aizawa of Department of Agricultural Chemistry, University of Tokyo, for the infrared work, Mrs. K. Sato, Miss I. Isobe and Miss M. Suzuki of Department of Agricultural Chemistry, University of Tokyo, for micro-analysis. We are also indebted to Kyowa Fermentation Industries Ltd. for kindly supplying us with crystalline gibberellin used in this work. The study was supported in part by the Grant in Aid for Agricultural Research from the Ministry of Education, to which the authors express their thanks.

---

8) T. Yabuta, Y. Sumiki, K. Aso, T. Tamura, H. Igarashi and K. Tamari, *J. Agr. Chem. Soc. Japan*, **17**, 894 (1941).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 418～427, 1959]

Studies on Growth Inhibition of Hiochi-bacteria, Specific Saprophytes of Saké

Part IV. Hydroxyaspergillic Acid as a Growth Inhibitant against Hiochi-bacteria*

By Seiji NAKAMURA** and Teruo SHIRO***

Yamamura Saké-Brewing Laboratory, Kobe

Received January 6, 1959

The authors have succeeded in isolating an antibiotic, from the cultured filtrate of *Asp. oryzae*, responsible for, at least, the majority of the active substances against hiochi-bacteria, as pale yellow crystals of m.p. 152～153°C. It has been proved that this antibiotic is identical with hydroxyaspergillic acid. The minimum concentration for complete inhibition in diluted Saké-peptone medium against true hiochi-bacilli, *Lactobacillus homohiochii* H-42 and *Lactobacillus heterohiochii* H-1, was 10 γ/ml, and that against hiochi-lactobacilli H-7 and H-34 20 γ/ml.

INTRODUCTION

Saké (Japanese rice-wine) is often spoiled by the action of a specific bacteria called "Hiochi-bacteria" which cause turbidity, and an undesirable smell and taste in Saké. In Japan, since far back, this phenomenon had been called "Hiochi". Hiochi-bacteria belong to the category of lactic acid bacteria. Takahashi[1], formerly, divided them into two groups, namely "hiochi-lactobacilli" and "true hiochi-bacilli". Recently, Kitahara et al.[2] carried out the identification and classification of hiochi-bacteria. The above authors divided true hiochi-bacilli into two groups of homo- and hetero-fermenters and proposed the names *Lactobacillus homohiochii* and *L. heterohiochii nov. sp.*, respectively, and also recognized that in hiochi-lactobacilli, strains of hetero-fermenter are identified as *Lactobacillus fermentum* α and those of homo-fermenter as *L. plantarum* and *L. acidphilus*, and only one strain as *Leuconostoc mesenteroides sp.*.

In regard of antiseptics of Saké, salicylic acid has been solely used for many years, but this acid can not inhibit the growth of hiochi-bacteria, satisfactorily. Recently, some antagonic substances of a growth factor for hiochi-bacteria have been found to be effective in preventing growth of hiochi-bacteria[3,4,5,6]. On the other hand, many antibiotics, such as penicillin, homomycin, mikamycin, chloramphenicol, actinomycin, aureomycin, catenulin, fradiomycin, leucomycin, gramicidin, polymixin, luteomycin, streptomycin etc. have been recognized to inhibit growth of hiochi-bacteria[7,8]. Growth inhibition of hiochi-bacteria in Koji*-extract of some strains of *Asp. oryzae* was first suggested by

---

[*] A short communication in reference of this work is published in *Bull. Agr. Chem. Soc. Japan*, **23**, 65 (1959).

[**] Present adress: Institute of Applied Microbiology of Univ. of Tokyo, Tokyo.

[***] Present adress: Department of Biology, Faculty of Science, Osaka University, Osaka.

[1] T. Takahashi, *Bull. Coll. Agr. Tokyo Imp. Univ.*, **7**, 531 (1907).

[2] K. Kitahara, T. Kaneko and O. Goto, *J. Gener. Appl. Microbiol.*, **3**, 102, 111 (1957).

[*] Koji; culture of Aspergilli on steamed rice.

[3] S. Fukui and Y. Tani, *J. Fermentation Technology*, **29**, 257 (1951); **31**, 4, 5 (1953).

[4] S. Tamura, G. Tamura, M. Takai, S. Nakamura and T. Shiro, This Bulletin, **22**, 202 (1958).

[5] S. Teramoto, W. Hashida and E. Yasuda, *J. Fermentation Technology*, **33**, 9, 403, 476 (1955).

[6] Y. Tani, *ibid.*, **36**, 360 (1958).

[7] Y. Inomoto, W. Hashida and A. Yamamoto, *ibid*, **29**, 204 (1951); **31**, 376 (1953).

[8] S. Nakamura and T. Shiro, *J. Agr. Chem. Soc. Japan*, **32**, 445 (1958).

Takahashi et al.[9] in 1932. Otani[10] successively reported that cultured filtrates of some strains of *Asp. oryzae* and *Pen. fluitans* inhibited growth of hiochi-bacteria. In 1951 Shimoda[11] reported on a strain of *Asp. oryzae* that produced an antibiotic substance against hiochi-bacteria, and isolated this antibiotic from the cultured filtrate of this strain, as white needle crystals, molecular formula, $C_8H_{13}NO_5$, m.p. 162∼163°C, and named it "Oryzacidin".

In the previous papers[12,13] the authors reported the results of a search for antibiotics active against hiochi-bacteria which was conducted by shaking culture among 314 strains of *Asp. oryzae*. The strains were at least divided into two groups: the cultured filtrates of some strains showed inhibition against the growth of true hiochi-bacilli but no inhibition against hiochi-lactobacilli whereas, on the contrary, cultured filtrates of the other 18 strains showed remarkable strong activity to the growth of hiochi-lactobacilli as well as true hiochi-bacilli.

The present paper deals with the isolation and properties of an antibiotic active against both hiochi-bacteria. The antibiotic compound, pale yellow needles, melted at 152∼153°C, having a formula of $C_{12}H_{20}N_2O_3$.

From the results of investigation of properties, reactions, spectra of this compound, it was proved that this antibiotic is identical with hydroxyaspergillic acid. Hydroxyaspergillic acid was first isolated by Menzel et al.[14] from cultured filtrate of a strain of *Asp. flavus* with the main product, aspergillic acid[15,16,17,18]. Hereupon, it was observed that the antibacterial activity of hydroxyaspergillic acid is only from one-tenth to one-third that of aspergillic acid. Quite recently, the structure (I) was established by Dutcher[19]. It has a very similar structure to aspergillic acid (II) expect an additional tertiary hydroxy group located at the position of the *sec*-butyl chain as shown in the following formula:

EXPERIMENTAL METHODS AND RESULTS

1) Determination of Antibiotic Activity against Hiochi-bacteria The ordinary dilution method which was the same as that reported in the previous papers[8,13] was used for the assay of the cultured medium, itself, or its solvent extracts, using true hiochi-bacillus H–1 and hiochi-lactobacillus H–34 as test organisms[2]. The assay medium used was Tamura's modified basal medium[20] such as described in Table I. Namely, for hiochi-lactobacilli, 5 g of polypeptone (P), but for true hiochi-bacilli, 5 g of P and 2 mg of *dl*-mevalonic acid* (M, synthesized, hiochic acid, a new growth-factor for true hiochi-bacilli[21,22]) were added to 1L of the basal medium. Preliminary culture of the test organisms was carried out in Matsuyama's Saké medium[23] containing 1% polypeptone for 7∼10 days at 30°C. As the procedure, 1 ml of a twofold concentration of the above basal medium described in Table I was added to each small test tube. After sterilization (120°C, at a moment) each tube was added with the sample and filled up with sterilized water to 2 ml, then, resterilized at 63∼65°C for five minutes. To this tube, one drop (0.03 ml; viable amount 10^4/ml, counted by Matsuyama's method[23]) of 7∼10 days' preculture of hiochi-bacteria was inoculated and then incubated at 30°C, for 10∼12 days. The acidity of the culture medium was determined by titration with a N/20 NaOH solution using bromthymolblue as the indicator.

2) Preparation of Culture Medium Although all of the 18 strains of *Asp. oryzae* produced antibiotic activity against hiochi-bacteria H–1 and H–34, *Asp. oryzae* A–0–4 was used as the representative strain. One hundred ml of the synthetic medium (glucose 50 g, polypeptone 20 g, KH_2PO_4 5 g, $CaHPO_4$ 2.5 g, $MgSO_4$ 2.5 g, dist. water 1 L, pH 5.2∼5.3) was taken in a 500 ml-volume flask, and after sterilization, inoculated with the spore suspension of strain A–0–4, and cultured on a reciprocating shaking machine at 27°C for 7 days.

---

[9] T. Takahashi and A. Suzuki, *ibid.*, **8**, 1064 (1932).

[10] Y. Otani, *J. Fermentation Technology*, **15**, 203 (1937.)

[11] C. Shimoda, *J. Agr. Chem. Soc. Japan*, **25**, 254 (1951).

[12] S. Nakamura, T. Shiro and N. Kunisada, *J. Agr. Chem. Soc. Japan*, **32**, 450 (1958).

[13] T. Shiro and S. Nakamura, *ibid*, **33**, 500 (1959).

[14] A. E. O. Menzel, O. Wintersteiner and G. Rake, *J. Bact.*, **46**, 109 (1943).

[15] E. C. White and J. H. Hill, *ibid.*, **45**, 433 (1943).

[16] C. R. D. Woodwards, *ibid.*, **54**, 375 (1947).

[17] J. D. Dutcher and O. Wintersteiner, *J. Biol. Chem.*, **155**, 359 (1944).

[18] J. D. Dutcher, *ibid.*, **171**, 321, 341 (1947).

[19] J. D. Dutcher, *ibid.*, **232**, 785 (1958).

* Kindly synthesized by Dr. S. Tamura.

[20] G. Tamura and A. Nagura, *J. Agr. Chem. Soc. Japan*, **32**, 701 (1958).

[21] D. Wolf, C. Hoffman, P. Aldrich, H. Skeggs, L. Wright and K. Folkers, *J. Am. Chem. Soc.*, **78**, 4499 (1956).

[22] G. Tamura, *J. Gener. Appl. Microbiol.*, **2**, 431 (1956); This Bulletin, **22**, 202 (1957).

[23] A. Matsuyama, *J. Agr. Chem. Soc. Japan*, **28**, 418, 422 (1954).

TABLE I. ASSAY MEDIUM FOR HIOCHI-BACTERIA

B:	Glucose	20 g	Adenine	10 mg
	Na-acetate	20	Guanine	10
	Fumaric acid	0.5	Uracil	10
	NH_4Cl	0.5	Xanthine	10
	Vitamin-B_1	1 mg	KH_2PO_4	500 mg
	〃 -B_2	1	K_2HPO_4	500
	〃 -B_6	1	$MgSO_4 \cdot 7H_2O$	200
	Nicotinic acid	1	$FeSO_4 \cdot 7H_2O$	10
	Pantothenic acid	1	$MnSO_4 \cdot 4H_2O$	10
	p-Amino-benzoic acid	200 γ	Tween-80	1 ml
	Biotin	10		
	Folic acid	10		
	Vitamin-B_{12}	10		
P:	Polypeptone	5 g		
M:	Mevalonic acid (Hiochic acid)			
	natural*	1 mg		
	(or synthesized**	2 mg)		

Total volume: 1 L. pH: 4.5~4.7

* Kindly supplied by Dr. G. Tamura, isolated from culture filtrate of *Asp. oryzae*.

** Kindly synthesized by Dr. S. Tamura.

TABLE II. EFFECTS OF THE CULTURED FILTRATES OF *Asp. oryzae* ON THE GROWTH OF HIOCHI-BACTERIA

Shaking Culture Age, days	4			7		
Representative Strains of *Asp. oryzae*	pH of Broth	Acidity of Assay Medium after 12 Days Incubation of Hiochi-bacteria (ml N/20 NaOH per 2 ml medium)		pH of Broth	Acidity of Assay Medium after 12 Days Incubation of Hiochi-bacteria (ml N/20 NaOH per 2 ml medium)	
		H-1	H-34		H-1	H-34
A-0-4	5.7	3.08	2.71	7.3	0.05	0.25
A-0-13	4.0	3.40	3.47	7.4	1.54	3.39
〃 14	3.4	3.07	3.58	7.7	1.20	3.93
A-0-25	5.2	0.06	0.05	7.3	0.11	0
〃 26	5.3	3.35	3.41	6.9	0	0.17
〃 27	4.8	4.49	3.51	7.4	0	2.00
〃 28	6.0	2.07	3.65	7.3	0.05	3.69
〃 29	6.2	0.81	3.46	7.6	0.64	3.78
〃 30	6.7	0.94	3.57	7.3	0	3.40
Control	—	0.50	3.69	—	0.50	3.69

Note: The strains of A-0-4, A-0-25, A-0-26 and A-0-27 showed inhibition against the growth of true hiochi-bacilli and hiochi-lactobacilli in the final stage of culture, however, only A-0-25 showed inhibition even in eary stage. A-0-28, A-0-29 and A-0-30 showed inhibition only against the growth of true hiochi-bacilli, but no inhibition against hiochi-lactobacilli. Although, A-0-13 and A-0-14 stimulated the growth of true hiochi-bacilli in both stage, they could not inhibit the growth of hiochi-lactobacilli in all stage of culture.

At an early stage of culture, the cultured filtrate accelerated the growth of hiochi-bacteria, because at this stage mevalonic acid, peptides and another growth factor of hiochi-bacteria were produced. But at the final stage, the antibiotic substance was produced in the medium, consequently, the cultured filtrate showed antibiotic activity against hiochi-bacteria (Table II).

3) Isolation of Antibiotic from the Culture Filtrate Two main types of extraction procedures were employed for the isolation of antibiotic from cultured medium, namely, the carbon process and the ether process.

a) Carbon Process: Seven liters of a cultured medium was acidified with hydrochloric acid to pH 2.5, stirred with 210 g of active carbon and filtered. The carbon washed with a small amount of water, was eluted with 600 ml of 80% aqueous acetone for four times. The eluates were combined and concentrated in vacuo, resulting 400 ml of rich water. The aqueous concentrate was adjusted to pH 2.5, and extracted four times with 400 ml of ether, solvent layers were concentrated in vacuo to dryness, yielding about 3 g of the crude antibiotic. For further purification, it was dissolved in 200 ml of ethyl acetate, and reextracted three times with 40 ml of 2% sodium bicarbonate solution. The aqueous layer was subsequently concentrated, adjusted to pH 2.5, and extracted with approx. 10 ml of chloroform for two of three times. The final chloroform extract was concentrated in vacuo to dryness, and about 2.5 g of a yellowish crude powder of antibiotic was obtained. After several recrystallizations from 50% aqueous ethyl alcohol and *n*-hexane, the melting point was found to be constant at 152～153°C. The yield of the crude crystal from 1L of the cultured medium was 300～500 mg.

b) Ether Process: This method differed from the carbon process only at the first stage where the primary concentration of the antibiotic was achieved by extraction with ether. Seven liters of a cultured medium were concentrated by vaporization in a flash evaporator to 700 ml. This aqueous concentrate was acidified with hydrochloric acid to pH 2.5, placed in a Soxlet extractor, and after extraction with ether for fourty hours, the solvent layer was concentrated in vacuo to dryness. About 3 g of the crude antibiotic was obtained. Further purification was carried out by the same procedure as the carbon process.

4) Chemical and Physical Properties This antibiotic is a pale yellow needle having a weak acidic character, m.p. 152～153°C, sublimes and it is readily soluble in methanol, ethanol, butanol, ethyl acetate, butyl acetate, chloroform, acetone, benzene, glacial acetic acid, slightly soluble in ether and *n*-hexane, but practically insoluble in petroleum ether and ligroin. Though it is highly insoluble in cold water, it is dissolved in alkaline solution.

The optical rotation of this antibiotic is $[\alpha]_D^{28}=+35.85°$ (c=0.3 in methanol). It does not contain sulfur, phosphorous, halogen and ash. Elementary analysis; Found: C, 59.93, H, 7.94, N, 11.51, O, 21.22%, M.W. 276 (as determined by the Rast method), and 259 (as determined by titration). Calcd. for $C_{12}H_{20}N_2O_3$: C, 60.00, H, 8.33, N, 11.66, O, 20.00%, Calcd. M.W. 240.

The ultraviolet spectrum (Fig. 1) in methanol showed absorption peaks at λ_{max} 330 mμ (ε 7500) and λ_{max} 232 mμ (ε 7900). The pKa value for this antibiotic was determined by potentiometric titration as shown in Fig. 2, pKa=5.15. It gave positive color reaction for ferric chloride solution (red-brown) and ninhydrin (red-violet) tests, but gave a negative Fehling test. However,

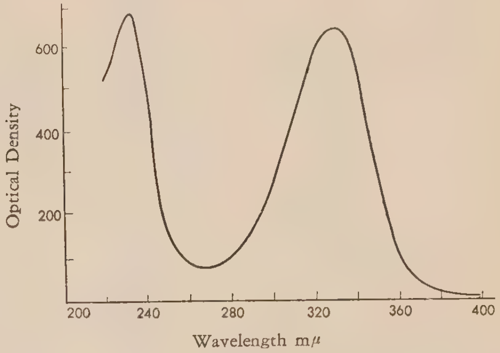
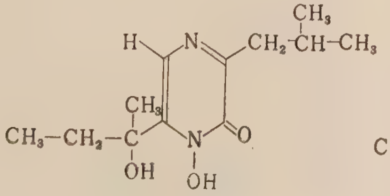
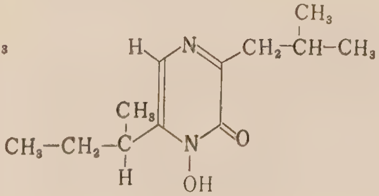


FIG. 1. Ultraviolet Absorption Spectrum of Antibiotic Compound



(I) Hydroxyaspergillic acid



(II) Aspergillic acid

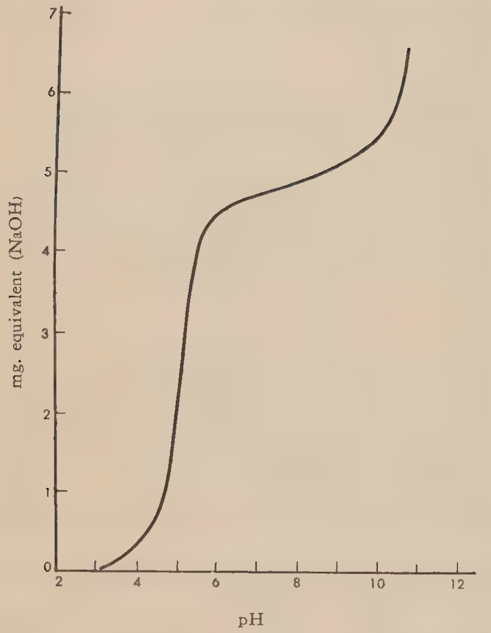


FIG. 2. Electronic Titration with Glass Electrode.

the redox reaction with Tollens reagent was positive. The iodoform test was negative. The primary amino group could not be observed by the micro-gasmetric method of Van Slyke.

5) Identity of Antibiotic to Hydroxyaspergillic Acid The formula of this antibiotic, $C_{12}H_{20}N_2O_3$, was coincident with hydroxyaspergillic acid and neohydroxyaspergillic acid[24]. The properties, reactions, and spectra of this compound showed strong resemblance to those of aspergillic acid. A summary of physical and chemical properties of aspergillic acid and its related antibiotics are given in Table III.

Taking the results of optical activity into account it seems likely that this antibiotic of m.p. 152~153°C is identical with hydroxyaspergillic acid. Moreover, identity was confirmed as follows by means of mixed melting point, accordance of infra red spectrum etc. of hydroxyaspergillic acid which was supplied through the courtesy of Dr. Dutcher.

a) Mixed Melting Point Test: A mixture with the hydroxyaspergillic acid sent from Dr. Dutcher (m.p. 148°C) melted at 148~149°C. Although the authors' sample a more or less higher mixed m.p. than Dr. Dutcher's sample, identity of this antibiotic with hydroxyaspergillic acid was confirmed from the fact that the mixed melting point was not depressed.

b) Infra Red Spectrum: The infra red spectra of this antibiotic and hydroxyaspergillic acid in Nujol mull are shown in Fig. 3. The identity of this antibiotic with hydroxyaspergillic acid was confirmed in accordance with their infra red spectra. A comparison of the infra red spectrum of aspergillic acid in Nujol mull is shown in Fig. 4, of which the sample has also kindly been sent us from Dr. Dutcher.

TABLE III. COMPARISON OF PHYSICAL AND GHEMICAL PROPERTIES OF AUTHORS' ANTIBIOTIC AND RELATED COMPOUND

	Authors' Antibiotic	Hydroxy-aspergillic acid[18,19]	Neohydroxy-aspergillic acid[24]	Aspergillic acid[14,15,17]
Appearance	pale yellow needles	pale yellow needles	colorless needles	yellow needles
M.p.	152~153°C	148~150°C	164~166°C	97~99°C
Formula	$C_{12}H_{20}N_2O_3$	$C_{12}H_{20}N_2O_3$	$C_{12}H_{20}N_2O_3$	$C_{12}H_{20}N_2O_2$
M.W.	240	240	240	224
Optical rotation	$[\alpha]_D^{28}=+35.85$ ethanol $c=0.3$	$[\alpha]_D=+36°$ ethanol $c=1.0$	$[\alpha]_D^{25}=-58°$ ethanol $c=1.01$	$[\alpha]_D^{24}=+13.4°$ ethanol $c=0.85$
pKa	5.15	4.9		5.5
Ultraviolet spectrum	$\lambda_{max}=330$ mμ $\varepsilon=7500$	$\lambda_{max}=328$ mμ $\varepsilon=2800$	$\lambda_{max}=328$ mμ $\varepsilon=8350$	$\lambda_{max}=328$ mμ $\varepsilon=8500$
	$\lambda_{max}=232$ mμ $\varepsilon=7900$	$\lambda_{max}=235$ mμ $\varepsilon=8750$	$\lambda_{max}=236$ mμ $\varepsilon=6450$	$\lambda_{max}=235$ mμ $\varepsilon=10500$

c) Paper Chromatography: The ascending paper chromatography of this antibiotic, Dr. Dutcher's hydroxyaspergillic acid and aspergillic acid was carried out simultaneously at room temperature. Filter paper; Toyoroshi No. 50, solvent; *n*-butanol saturated with 0.5% ammonia, the ascending technique for eighteen hours at room temperature. For development of the spots, a ferric chloride solution was used. This anti-

---

[24] U. Weiss, F. Strelitz, H. Flow and I. N. Asheshov, *Arch. Biochem. and Biophys.*, **74**, 150 (1958).

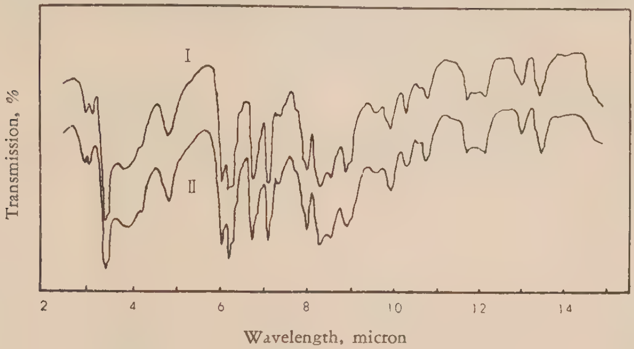


FIG. 3. Infra Red Spectra of Authors' Antibiotic and Dr. Dutcher's Hydroxyaspergillic Acid.

I: Authors' sample
II: Dr. Dutcher's hydroxyaspergillic acid

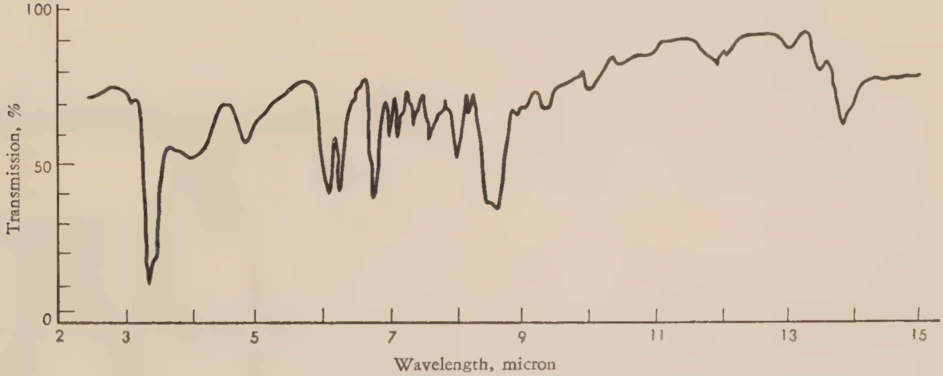


FIG. 4. Infra Red Spectrum of Aspergillic Acid

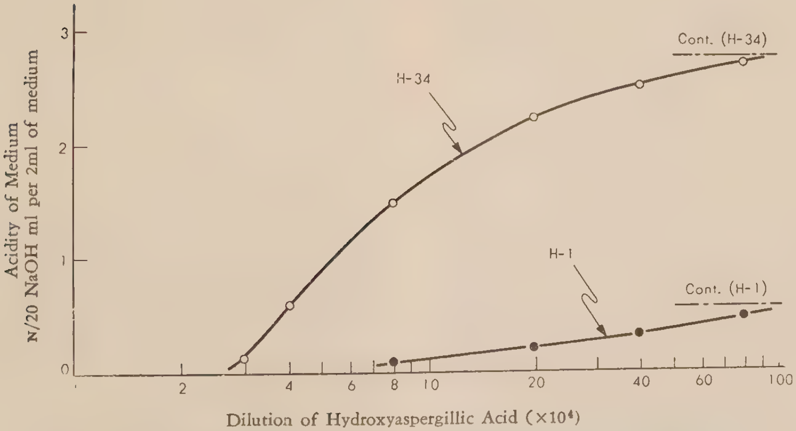


FIG. 5. Antibiotic Activity of Hydroxyaspergillic Acid against Hiochi-bacteria in Modified Tamura's Medium.

TABLE IV. ANTIBIOTIC POTENCY OF HYDROXYASPERGILLIC ACID IN MATSUYAMA'S DILUTED SAKÉ MEDIUM

	Hiochi-bacteria				
Dilution	True hiochi-bacilli		Hiochi-lactobacilli		
($\times 10^4$)	H-1	H-42	H-7	H-34	T-101
1	− −	− −	− −	− −	− −
2	− −	− −	− −	− −	− −
5	− −	− −	− −	− −	− −
8	− −	− −	± −	±、±	++ ++
10	− −	− −	+ +	++ ++	++ ++
16	+ +	+ +	++ ++	++ ++	+++ +++
Control	+ +	+ +	++ ++	++ ++	+++ +++

(−) ; no growth.
(±) ; slightly cloudy.
(+) ; slightly growth.
(++) ; growth more than 10^6/ml. (viable count*)
(+++) ; growth more than 10^9/ml. (viable count*)
* counted by Matsuyama's method[28].

biotic and Dr. Dutcher's hydroxyaspergillic acid showed the same value, namely, $R_F=0.91$, while that of aspergillic acid showed 0.95.

From results of the above experiments, it should be concluded that this antibiotic is identical with hydroxyaspergillic acid.

6) Biological Properties

a) Antibiotic Activity against Hiochi-bacteria: The antibiotic activity of hydroxyaspergillic acid was tested by the dilution method as described above. The activity against true hiochi-bacillus H-1 and hiochi-lactobacillus H-34 in Tamura's modified basal medium (Table I) is shown in Fig. 5, namely, the growth curve for 12 days' culture shown in Fig. 5 indicated that hydroxyaspergillic acid is bacterio-static for these hiochi-bacteria at a concentration of 1 : 30,000 (33 γ/ml) and that at concentrations lower than 1 : 40,000 it is almost ineffective for the growth of hiochi-bacteria.

A study on the effect of hydroxyaspergillic acid in Matsuyama's diluted Saké medium[23] (composed of 70-parts of Saké, 30-parts of water, and 1-part of polypeptone, containing 16～17% alcohol) upon the growth of hiochi-bacteria was carried out by incubation for 10 days at 30°C. In this test, growth of hiochi-bacteria was observed by turbidity. The minimum inhibitory concentration for hiochi-bacteria changes more or less according to the quality of the Saké used in the medium. However in this case minimum inhibitory concentration against true hiochi-bacilli (*Lactobacillus homohiochii* H-42 and *L. heterohiochii* H-1) was 10 γ/ml and that against hiochi-lactobacilli (H-7, H-34), 20 γ/ml. The growth of Teramoto's hiochi-bacteria T-101[25] was inhibited at the same concentration. The results of this test are shown in Table IV.

The valuation of hydroxyaspergillic acid as an antiseptic for Japanese Saké was carried out upon several types of brewered Saké. In this case, the alcohol content of each Saké tested (containing no antiseptics) was adjusted to 14% by dilution, because this level is optimum for the growth of hiochi-bacteria. Hiochi-bacteria, H-1, H-34 and T-101 were inoculated by the same procedure as described above. Although the effects of this antibiotic upon each kinds of Saké were not similar, hydroxyaspergillic acid showed activity against hiochi-bacteria at the dilution of 1 : 80,000 in diluted Saké as shown in Table V. It was concluded that hydroxyaspergillic acid was capable of being used as an antiseptic for Saké at least, in the concentration of 12～13 γ/ml.

b) Effect of Polypeptone and Mevalonic Acid upon the Activity of Hydroxyaspergillic Acid: As mentioned above, the potency of hydroxyaspergillic acid against hiochi-bacteria on Japanese Saké varies to some extent according to the quality of Saké. In Japanese Saké, mevalonic acid (hiochic acid) and peptides are naturally contained as growth factors of hiochi-bacteria, therefore, the effects of polypeptone and mevalonic acid upon the

---

[25] S. Teramoto, W. Hashida and D. Yoshino, *J. Fermentation Technology*, **32**, 180 (1954).

TABLE V. ANTIBIOTIC ACTIVITY AGAINST HIOCHI-BACTERIA IN VARIOUS SAKÉ

Saké	Dilution ($\times 10^4$)	Hiochi-bacteria					
		H-1		H-34		T-101	
		(Culture age, days)					
		21	30	21	30	21	30
A	2	−	−	−	−	−	−
	4	−	−	−	−	−	−
	8	−	±	−	±	±	±
	16	±	+	+	+	+	+
	cont.	+	+	+	+	+	+
B	2	−	−	−	−	−	−
	4	−	−	−	−	−	
	8	−	−	−	−	−	±
	16	−	+	−	+	+	+
	cont.	−	+	+	+	+	+
C	2	−	−	−	−	−	−
	4	−	−	−	−	−	−
	8	−	−	−	−	−	±
	16	−	+	±	+	+	+
	cont.	+	+	+	+	−	+
D	2	−	−	−	−	−	−
	4	−	−	−	−	−	−
	8	−	±		+	+	+
	16	+	+	+	+	+	+
	cont.	+	+	+	+	+	+
E	2	−	−	−	−	−	−
	4	−				−	−
	8	−	+	−	+	−	−
	16	−	+	+	+	+	+
	cont.	±	+	+	+	+	+
F	2	−	−	−	−	−	−
	4	−	−	−	−	−	−
	8	−	−	−	−	−	−
	16	−	±	−	±	−	±
	cont.	−	+	−	+	−	+

Note: The growth of hiochi-bacteria was observed by the same method as described in Table IV.

activity of hydroxyaspergillic acid were observed on the basal medium for hiochi-bacteria. As shown in Figs. 6 and 7, the activity of hydroxyaspergillic acid was not affected by the addition of mevalonic acid, however, its activity was competitively inhibited by the presence of polypeptone in the medium. Detailed data concerning this problem will be published in the near future.

c) Biological Activity of Hydroxyaspergillic Acid against the Use of Lactobacilli in Saké-brewing: Since some species of the Lactobacilli26) are known as important acidifiers in the practical cultivation of "Moto"27), the yeast starter for Saké-brewing, the effect of hydroxyaspergillic acid upon growth of *Leuconostoc mesenteroides* and *Lactobacillus saké* was observed. But, almost no inhibition to growth of these strains was recognizable.

---

26) H. Katagiri and K. Kitahara, *J. Agr. Chem. Soc. Japan*, **10**, 942, 952, 959, 964 (1934).

27) O. Sugita, N. Kunisada and K. Kageyama, *J. Fermentation Technology*, **34**, 138 (1956).

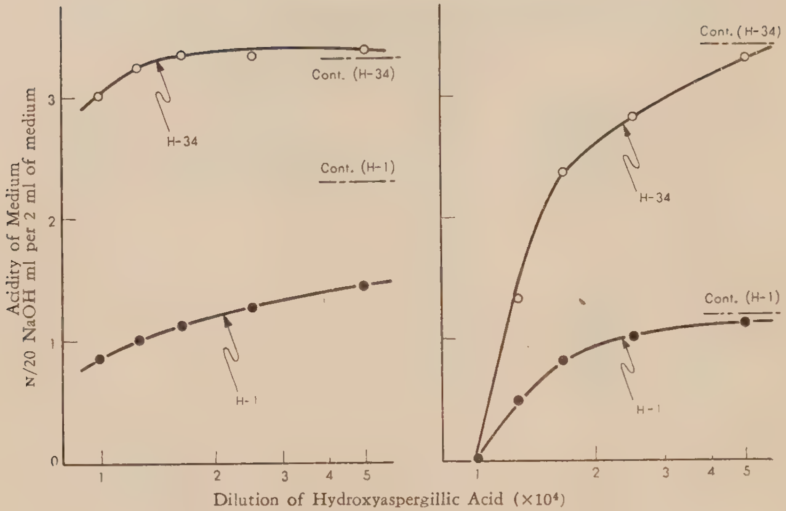


FIG. 6. FIG. 7.

FIG. 6 and 7. Effects of Polypetone and Mevalonic Acid upon the Activity ot Hydroxyaspergillic Acid.

Note: In Fig. 6, 20 g of polypeptone (P) and 2 mg of mevalonic acid (M) were added to 1 L of basal medium, namely, in this test P was contained as much as four times of standard basal medium in Table I. In Fig. 7, 10 g of P and 4 mg. of M were added to 1 L of basal medium, namely, in this test P and M contained as much as twice of standard medium in Table I. The growth of hiochi-bacteria (H-1, H-34) was observed by titration method of acidity.

TABLE VI. ANTIBIOTIC ACTIVITY OF HYDROXYASPERGILLIC ACID FOR LACTOBACILLI IN SAKÉ-BREWING

Dilution	N/20 NaOH ml/2 ml of medium	
(×10⁴)	*Leuconostoc mesenteroides*	*Lactobacillus saké*
1	2.34	2.60
2	2.39	2.76
4	2.41	2.91
8	2.46	2.79
16	2.37	3.01
control	2.55	3.05

Medium; B+P in Table I, but pH 5.5.

The results are summarized in Table IV.

d) Other Biological Effects of Hydroxyaspergillic Acid: Antimicrobial activity of hydroxyaspergillic acid was tested by application of the agar dilution method. But, growth of *Bac. subtilis*, *E. coli*, *Staph. aureus*, *Proteus vurgalis*, *Salmonella tim*, *Mycobact.* 607, *Pen. chrysogenum*, *Asp. niger*, *Can. albicans*, *S. cereviciae*, *T. utilis* and *xerosis* was not inhibited, at a concentration of 1 : 1,000. *Corynebact.*

e) Toxicity: Toxicity of hydroxyaspergillic acid was tested with mice weighting 15~20 g. The $\mathrm{L_{D50}}$, administered intravenous injection was 101 mg/Kg of body weight and that of intraperitoneal injection 90~100 mg/Kg.

CONCLUSION AND SUMMARY

From cultured filtrates of some strains of *Asp. oryzae*, an antibiotic which showed activity against true hiochi-bacilli and hiochi-lactobacilli was isolated, as a pale yellow needle having a weak acidic character, m.p. 152~153°C. Taking its physical and chemical properties into account it seems likely that this antibiotic is identical with hydroxyaspergillic acid, and identity was confirmed by mixed melting point and accordance of the infrared spectrum. The minimum concentration for complete inhibition in Tamura's modified basal medium was $33\,\gamma$/ml against hiochi-bacteria and concentrations of diluted Saké-peptone medium (composed of 70-parts of Saké, 30-parts of water, and 1-part of polypeptone) against true hiochi-bacilli (H-1, H-42) and hiochi-lactobacilli (H-7, H-34) were $10\,\gamma$/ml and $20\,\gamma$/ml, respectively. This is accounted for hydroxyaspergillic acid which as antiseptic of Japanese Saké, was capable of being used at least at the concentration of $12 \sim 13\gamma$/ml. On the contrary, this acid showed hardly any inhibition on growth of Lactobacilli which serviceable Saké-brewing. Though antibiotic activity of this substance against hiochi-bacteria was not affected by the addition of mevalonic acid, however, it was competitively inhibited by the presence of peptides. L_{D50}, for mice administered intravenously was about 100 mg/kg of body weight.

Acknowledgements The authors wish to express their sincerest thanks to Prof. Y. Sumiki of Univ. of Tokyo for his kind guidance and encouragement throughout the course of this work, and also to Emerritus Prof. K. Sakaguchi for his sound advice and to Dr. D. Dutcher of the Squibb Institute for Medical Research for a generous gift of the samples used here. They are also indebted to Prof. K. Kitahara of Univ. of Tokyo, and Prof. S. Teramoto of Univ. of Osaka for kindly transfering their cultures of hiochi-bacteria and *Leuconostoc mesenteroides* and *Lactobacillus saké*. Thanks are also due to Dr. S. Tamura, Dr. Y. Yonehara, Dr. G. Tamura, Dr. N. Takahashi, Dr. S. Marumo, Dr. K. Takamiya of Dept. of Agr. Chemistry, Univ. of Tokyo and Dr. H. Tani of Univ. of Osaka for their valuable advice and cordial assistance in this work.

The micro analysis, infra red spectra were carried out at Dept. of Agr. Chemistry, Univ. of Tokyo, and animal experimental tests were performed at the Institute of Applied Microbiology of Univ. of Tokyo and Kyowa Fermentation Industry Co. Ltd..

Acknowledgement is made to Mr. T. Yamamura, President of their company, for his encouragement in this work and members of this laboratory for their kind advice.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 428～437, 1959]

Islanditoxin, a Toxic Metabolite produced by *Penicillium islandicum* Sopp

Part III. Structure of Islanditoxin

By Shingo Marumo*

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo

Received January 13, 1959

Islanditoxin is a novel peptide consisted of five amino acids, three of which have been known as normal components of proteins and the remaining two, D-β-phenyl-β-aminopropionic acid and L-dichloroproline have not been encountered previously in nature. The structure of islanditoxin is shown to be a cyclic polypeptide, represented by formula (VIII).

The isolation of islanditoxin, a toxic peptide, from a culture broth of *Penicillium islandicum*, which caused the islandia yellowsis rice, was described in the previous papers[1,3], and the general characterization and acid hydrolysis of islanditoxin were also reported[2,4]. In this paper the author wishes to report the correction of molecular formula, some physical properties and chemical structure of islanditoxin as well as the existence of two novel amino acids, D-β-phenyl-β-aminopropionic acid and L-dichloroproline. On the basis of degradation experiments the structure of islanditoxin is proposed as (VIII).

When islanditoxin was recrystallized from methanol, isladitoxin monomethanolate, m.p. 250～1°, was obtained in colourless needles having one mole of methanol as the solvent of crystallization. Using water as the solvent, one mole of water was taken and islanditoxin monohydrate, m.p. 254°, was formed. The solvent of crystallization, methanol and water, were combined strongly with islanditoxin, and were not lost by vacuum-drying at 100° for three days and so by drying at 146° (using tetrachloroethane) for three days. To obtain free islanditoxin, it was needed to recrystallize the monomethanolate or monohydrate from boiling acetone. On cooling the solution free islanditoxin, m.p. 258°, deposited in an amorphous form. The solubility of free islanditoxin was different from that of the monomethanolate and almost insoluble in ordinary solvents except phenol and glacial acetic acid. The molecular formula, microanalyses and infrared spectra of them (as Nujol mull) are shown in Fig. 1.

It is noted that the different among their infrared spectra is observed in the ranges of 3500～3300 and 1100～1000 cm^{-1}. corresponding to OH regions. Free islanditoxin, when recrystallized from methanol or water, forms the monomethanolate or monohydrate and this conversion is reversible if proper solvents are used.

The paper chromatography was carried out in order to examine the homogeneity of islanditoxin. On paper chromatograms only one spot was detected, hence islanditoxin is considered to be chromatographically a pure substance.

On the complete acid hydrolysis of islanditoxin[4] there were obtained three ninhydrin possitive substances, two of which were identified as serine and α-aminobutyric acid respectively

---

* Present address: The Institute of Physical and Chemical Research, Komagome Bunkyo-ku, Tokyo.

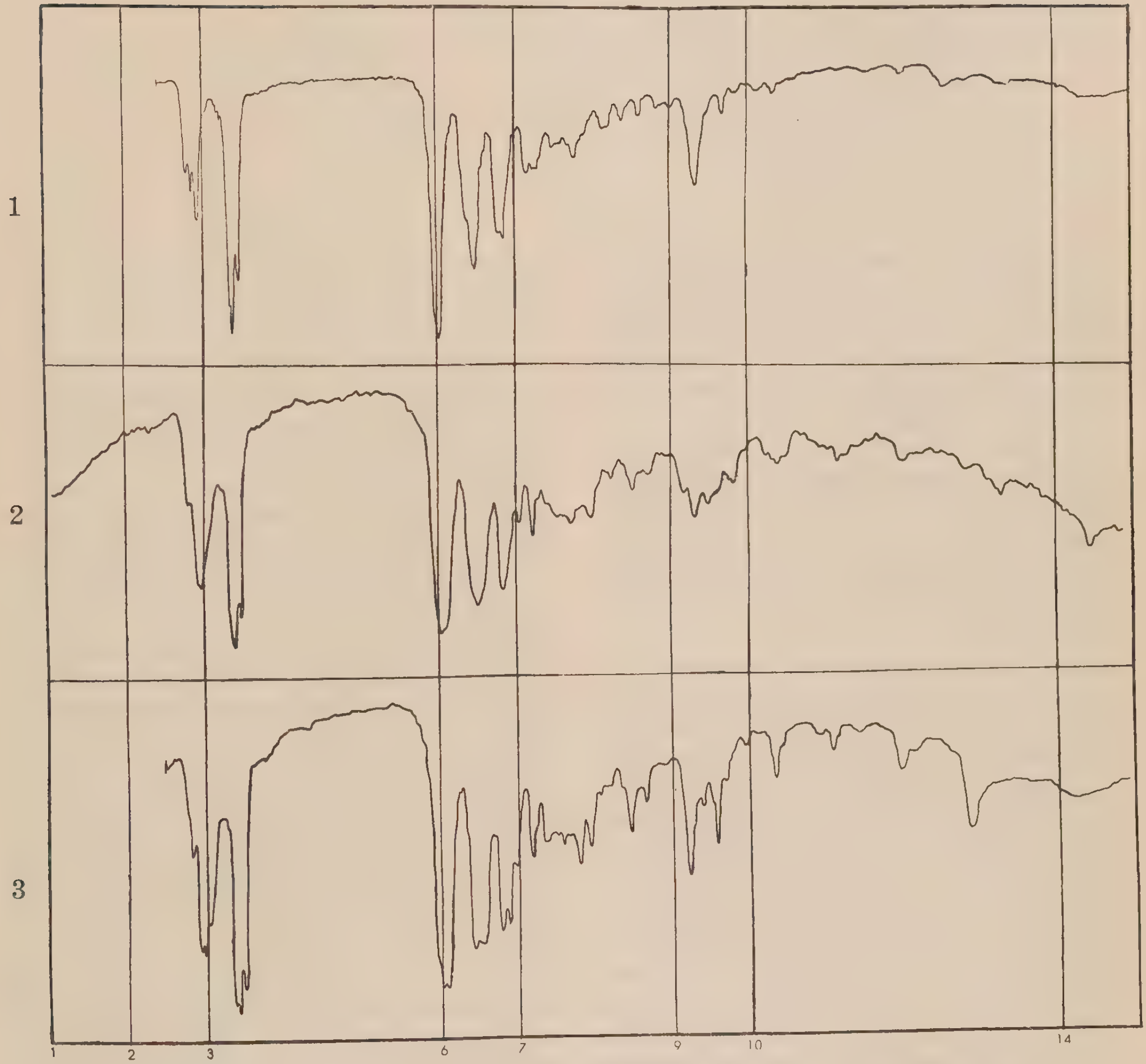
1) S. Marumo and Y. Sumiki, *J. Agr. Chem. Soc. Japan*, **29**, 305 (1955).

2) S. Marumo, K. Miyao and A. Matsuyama, *ibid*., **29**, 913 (1955).

3) S. Marumo, This Bulletin, **19**, 258 (1955).

4) S. Marumo, K. Miyao and A. Matsuyama, *ibid*., **19**, 262 (1955).

FIG. 1.



1. $C_{24}H_{31}O_7N_5Cl_2$

Calcd. C, 50.35; H, 5.45; N, 12.23; Cl, 12.40.
Found. C, 50.68; H. 5.28; N, 12.28; Cl, 12.51.

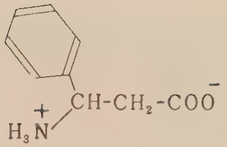
2. $C_{24}H_{31}O_7N_5Cl_2 \cdot CH_3OH$

Calcd. C, 49.67; H, 5.80; N, 11.59; Cl, 11.75.
Found. C, 49.81; H, 5.61; N, 11.85; Cl, 11.58.

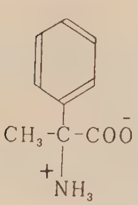
3. $C_{24}H_{31}O_7N_5Cl_2 \cdot H_2O$

Calcd. C, 48.81; H, 5.60; N, 11.86; Cl, 12.03.
Found. C, 48.86; H, 5.21; N, 11.81; Cl, 12.28.

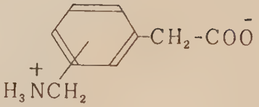
on the basis of their infrared spectra, R_F values and melting points. The third amino acid, $C_9H_{11}NO_2$, m.p. 230° (decomp.), had infrared absorptions at 1600, 1573, 1510, and 700 cm^{-1} indicating the presence of benzene ring. The ultraviolet absorption spectrum was found to be typical for the benzenoid, having maximum at 257 mμ (ε, 220). On the paper chromatograms, R_F value was a little higher than those of DL-phenylalanine and it was considered that the compound could be an amino acid which had been unknown in nature. Quantitative deter-



(I)



(II)



(III)

mination of serine and α-aminobutyric acid was carried out by paper chromatography with acid hydrolyzate of islanditoxin. As the result, it was revealed that one mole of islanditoxin liberated two moles of serine and one mole of α-aminobutyric acid.

The optical rotation measurement of serine obtained by acid hydrolysis gave $[\alpha]_D^{25°}=+15.4°$ (*c*, 3.5 in 1 N-hydrochloric acid) showing that the amino acid was of L-series. α-Aminobutyric acid was not oxidized by D-amino acid oxidase, hence the amino acid was found to be of L-series.

From the data obtained by acid hydrolysis, in islanditoxin there were two moles of L-serines, one mole of L-α-aminobutyric acid and one mole of $C_9H_{11}NO_2$ and the sum of the molecular formulae of these four molecules makes $C_{19}H_{26}O_8N_4$. Substructing it from the molecular formula of islanditoxin, there remains $C_5H_5NOCl_2$ as an unknown fragement which could not be obtained by acid hydrolysis but could be obtained by alkaline hydrolysis as described below.

Treatment of islanditoxin with saturated hot aqueous solution of barium hydroxide at 130° for twenty hours, gave rise to two kinds of organic acids in addition to the above amino acids. These acids were separated respectively by submitting the alkaline hydrolyzate on the celullose column chromatography.

One of the organic acid, $C_5H_5NO_2$, m.p. 180° (decomp.), having ultraviolet absorption maximum at 261 mμ (ε, 1.6×10^4), was identified as α-pyrrole carboxylic acid by usual methods comparing with an authentic sample, which was synthesized by Zugravescu's method[5]. It was presumed that this acid was originated from the unidentified fragement, $C_5H_5NOCl_2$.

The second organic acid, $C_8H_8O_2$, m.p. 130°, having ultraviolet absorption maximum at 272 mμ (1.6×10^4), was identified as cinnamic acid. As to the origin of cinnamic acid in the alkaline hydrolyzate of islanditoxin, the author considers it to be reasonable to assign the amino acid, $C_9H_{11}NO_2$, which is deaminated by the action of OH ion to produce cinnamic acid. From the above result, it is possible to propose the structure of the amino acid, $C_9H_{11}NO_2$, as being formula (I).

This amino acid had the following properties, a) from the infrared absorption at 3100 cm^{-1} (-NH$^+$) and 1570 cm^{-1} (-COO$^-$) and the behaviors on filterpaper electrophoresis this was a neutral amino acid of zwitter ion type. b) the intensity of ninhydrin colouration was weaker than those of normal α-amino acids and the developement of the spot changed instantaneously through yellow to purple. This is the characteristic feature of such a compound as benzylamine type and it means that phenyl and amino radicals attach to the same carbon atom[6]. c) the ultraviolet spectrum had maximum absorption at 257 mμ (ε, 220), indicating a typical benzene analogue substituted with alkyl groups, in which both amino and carboxyl radicals do not attach directly to benzene ring. The isomers, having the molecular formula of $C_9H_{11}NO_2$ and satisfying above three conditions, can be written in the three types as shown above, namely, β-phenyl-β-aminopropionic acid (I), α-phenyl-α-aminopropionic acid (II) and aminomethyl phenylacetic acid (III). Among these,

---

5) I. Zugravescu, *Bull. Soc. Chim.*, (5), **5**, 44 (1952).

6) M. Sawai et al., *J. Chem. Soc. Japan*, **76**, 256 (1955).

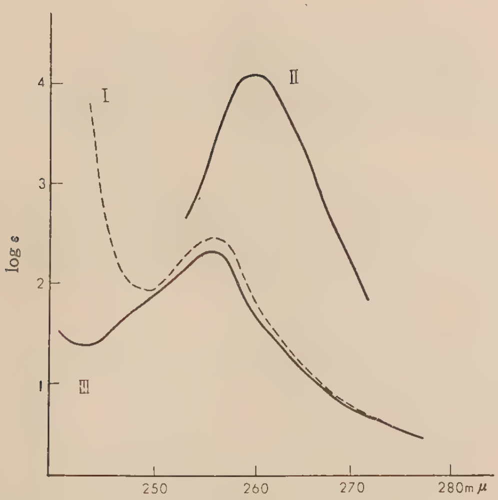


FIG. 2.

I, islanditoxin 257 mμ ε, 307
II, α-pyrrole carboxylic acid 261 mμ ε, 1.6×10^4
III, D-β-phenyl-β-amino propiomic acid 257 mμ ε, 220

the isomer which has the possibility to change into cinnamic acid, is exclusively the β-phenyl-β-aminopropionic acid. The fact that on the alkaline degradation $C_9H_{11}NO_2$ as well as islanditoxin produced cinnamic acid supported formula (I) as the structure of this amino acid. There was prepared DL-β-phenyl-β-aminopropionic acid⁷⁾ and solved into an optical antipode through the brucine salt of DL-formyl derivative⁸⁾. The amino acid in the hydrolyzate was found to be identical with a synthetic D-form by comparison of infrared spectra, chromatographic behavior and melting point. Moreover, measurement of the optical rotation of the amino acid showed $[\alpha]_D^{25°}=-9.6°$ (c, 5.0, in 1 N-potassium hydroxide), which was in agreement with the value reported by Fisher⁸⁾, hence (−) β-phenyl-β-aminopropionic acid in islanditoxin was proved to be of D-series.

The remaining problem is to determine the chemical structure of the chlorine-containing amino acid.

As described above, α-pyrrole carboxylic acid was obtained on the alkaline hydrolysis of islanditoxin. It is probable that α-pyrrole carboxylic acid was originated from the unidentified component, $C_5H_5NOCl_2$ of the islanditoxin molecule.

As shown in Fig. 2, the ultraviolet absorption spectrum of islanditoxin corresponds to that of β-phenyl-β-aminopropionic acid. The ε value of α-pyrrole carboxylic acid is too large to consider that the acid is a component of islanditoxin. It is reasonable to assume that the origin of α-pyrrole carboxylic acid in islanditoxin is dichloroproline, which had no absorption in ultraviolet region and is easily decomposed to α-pyrrole carboxylic acid, liberating two moles of hydrogen chloride by alkali. Dehydrochloroislanditoxin (V), $C_{24}H_{29}O_7N_5\cdot CH_3OH$, dehydrochloroislanditoxinic acid (VI), $C_{24}H_{31}O_8N_5\cdot H_2O$ and dehydrochloroislanditoxinic amide, $C_{24}H_{32}O_7N_6\cdot H_2O$, all had strong absorption at 268 mμ showing the formation of the pyrrole ring.

When islanditoxin was hydrogenated by PtO_2 at 130° for 8 hours at 180 atoms in ethanol solution and then decomposed by acid, proline was obtained in about 20% yield in addition to the three kinds of amino acids, which was separated with celullose column chromatography. From the result of comparison of the quantitative data obtained chemically and biologically, it was concluded that the proline belongs to of L-series. It was reasonable to consider that L-dichloroproline in islanditoxin was dechlorinated through catalytic reduction to be converted to L-proline. The positions of chlorine atoms attached to pyrrolidine ring and the configuration concerned are yet undetermined and the investigation is now underway.

The next problem is to determine the amino acid sequence of the islanditoxin. Evidences that islanditoxin is a cyclic peptide are given as follows. a) In the infrared spectrum of islanditoxin there are shown no peaks corresponding to $-NH^+$ and $-COO^-$. b) No DNP-islanditoxin is prepared by an ordinary method, showing the absence of free amino radicals. c) No C-terminal amino acid is obtained by hydrazinolysis of islanditoxin. d) By acetylation with acetic anhydride and pyridine the diacetyl is-

---

7) Org. synth. Coll. vol. **3**, 26.
8) E. Fisher, *Berichte*, **44**, 3166 (1911).

landitoxin is prepared, which should be attributed to two hydroxyl groups of two moles of serine, comfirming no other acetylating function suth as -NH_2 is absent. e) With steam distillation of islanditoxin hydrolyzate neither volatile acid nor amine are obtained. f) The molecular formula of islanditoxin is explained satisfactorily as a polypeptide consisting of five amino acid components. Consequently, islanditoxin is concluded to belong to a cyclic peptide.

The sequence of amino acid was established by partial hydrolysis. Preliminary experiments with enzymatic degradation using carbopeptidase and protease were unsuccessful. A search for the optimum condition for partial hydrolysis was first made by comparing the results of paper chromatography of the hydrolyzate and it was found that partial hydrolysis at room temperature using concentrated hydrochloric acid was the good condition.

The ninhydrin positive spot, R_F 0.75, after hydrolysis for 17 hours, was first detected. This spot was, on standing for further 6-13 days successively, divided into two spots, one of which was assigned to serine and the other to the further degradative peptide, R_F 0.59. Now the first spot was termed peptide A and the second termed peptide B. Peptides A and B were separated by a celullose column chromatography. The N-terminal amino acids of peptides A and B were determined by DNP-method and their C-terminal amino acids by hydrazinolysis. As the results, peptides A and B were illustrated as follows showing that both have the same N- and C-terminal amino acids.

$$\begin{array}{l}\text{Peptide A}\\ \text{(N) serine–}\end{array}\left[\begin{array}{l}\text{serine}\\ \text{dichloroproline}\\ \beta\text{-phenyl-}\beta\text{-amino}\\ \text{propionic acid}\end{array}\right]\begin{array}{c}\text{–}\alpha\text{-aminobutyric (C)}\\ \text{acid}\end{array}$$

$$\begin{array}{l}\text{Peptide B}\\ \text{(N) serine–}\end{array}\left[\begin{array}{l}\text{dichloroproline}\\ \beta\text{-phenyl-}\beta\text{-amino-}\\ \text{propionic acid}\end{array}\right]\begin{array}{c}\text{–}\alpha\text{-aminobutyric (C)}\\ \text{acid}\end{array}$$

Combining the above two schemes and taking the cyclic peptide into account, islanditoxin should be written as below.

$$\text{serine-serine–}\left[\begin{array}{l}\text{dichloroproline}\\ \beta\text{-phenyl-}\beta\text{-amino-}\\ \text{propionic acid}\end{array}\right]\begin{array}{c}\text{–}\alpha\text{-aminobutric}\\ \text{acid}\end{array}$$

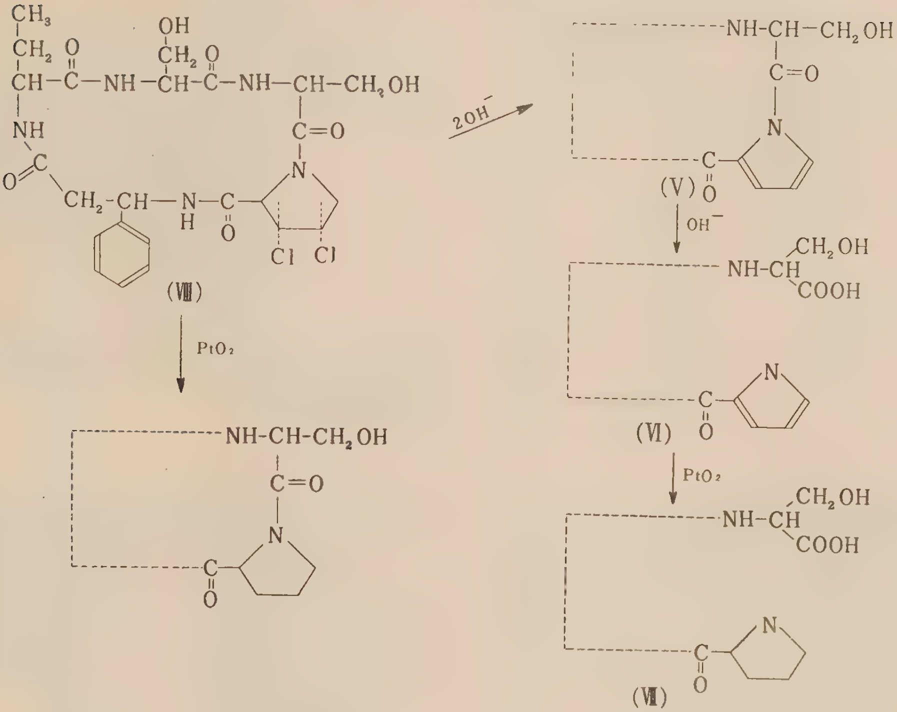
In the above scheme, the sequence of L-dichloroproline and D-β-phenyl-β-aminopropionic acid is yet obscure and could not be determined by the acid hydrolysis, but could be by the following experiments.

Dehydrochloroislanditoxinic acid (VI), $C_{24}H_{31}O_8N_5 \cdot H_2O$, U.V. max. 268 m$\mu$ (ε, 1.2×10^4), which was prepared under the limited condition of reacting exactly three moles of alkali to islanditoxin, was consisted of L-serine as its C-terminal amino acid, but N-terminal amino group did not be detected by DNP-method. If the N-terminal amino acid of dehydrochloroislanditoxinic acid would be originally dichloroproline and converted to pyrrole ring by alkali, it was well understood that islanditoxin was changed to dehydrochloroislanditoxinic acid, by consuming three moles of OH^-, liberating two moles of hydrogen chloride and splitting the peptide bond between pyrrole carboxylic acid and serine. To prove this assumption the dehydrochloroislanditoxinic acid (VI) was hydrogenated by PtO_2 in glacial acetic acid. The hydrogenation of pyrrole ring was not proceed quantitatively under the conditions applied. The hydrogenated product (VII) was sensitive to ninhydrin and developed yellow colour. The product was also sensitive to isatine and developed intense blue, which is characteristic toward pyrrolidine and piperidine9). These data showed that the N-terminal amino acid of (VII) was proline and that of (VI) was α-pyrrole carboxylic acid.

On the basis of above degradation experiments, amino acid sequence was made clear and the structure of islanditoxin is proposed as L-seryl-L-seryl-L-dichloroprolyl-D-β-phenyl-β-aminopropionyl-L-α-aminobutyric acid anhydride (VIII).

---

9) Traube et. al., *Ber.*, **56**, 1861 (1923)



EXPERIMENTAL

Paper Chromatography of Islanditoxin

Toyo filter paper No. 51 (30×400 mm) was used. The paper spotted with the sample was submitted to run about 30 cm long in the ascending method. The developed paper was dried well in an oven at 60°, and sprayed with N/10-alcohlic potassium hydroxide. After standing to dryness the paper was kept about 15 minutes in an atomosphers of chlorine gas formed by 1 N-hydrochloric acid and potassium permanganate. A desiccator was used conveniently for this purpose. The paper was taken out from the desiccator and stood until completely free from chlorine gas. (It was needed about twenty minutes.) Then it was inserted into a freshly prepared mixture of equal volumes of 1% of benzidine in 10% acetic acid and 1% potassium iodide. The spot of peptide developed immediately blue colour. By Sanger et al.[10], the solution of 1% iodine and 1% potassium iode and starch were used instead of above reagents. With islanditoxin preliminary treatment of papers with alkali was very effective fort he colouration. Solvent systems used and R_F values was listed in Table I.

TABLE I.

Solvent Systems	R_F values
methyl ethyl ketone : acetone : water (20 : 2 : 5)	1.0
water saturated *n*-butyl alcohol	0.84
methyl isobutyl ketone : acetone : methanol : water (30 : 3 : 10 : 10)	0.71
methyl isobutyl ketone : acetone : water (60 : 18 : 5)	0.63
methyl isobutyl ketone : acetone : water (5 : 1 : 15)	0.19
chloroform : methanol : water (5 : 1 : 15)	0

Quantitative Analysis of α-Aminobutyric Acid

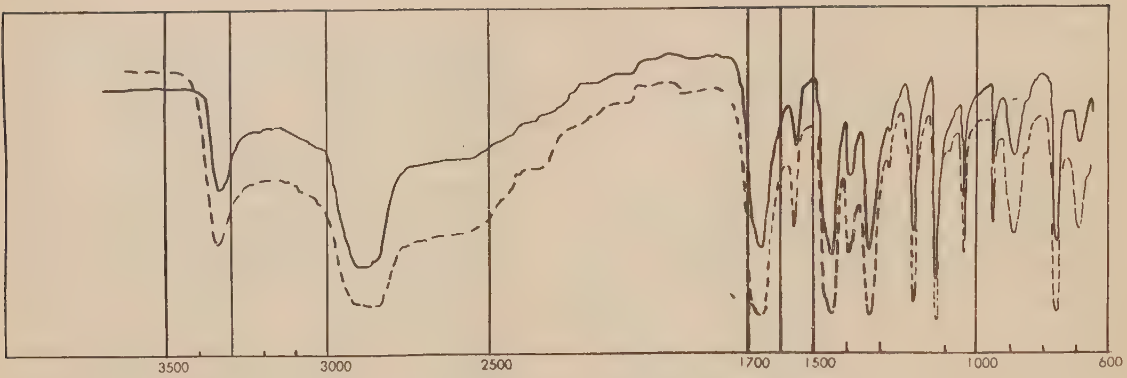
The analysis was made according to the quite similar method for serine estimation[4]. The concentration of hydrochloric acid used was 6 N. 0.86 mole of α-aminobutyric acid per one mole of islanditoxin was obtained on the hydrolysis for 20 hours.

Isolation of α-Pyrrole Carboxylic Acid and Cinnamic Acid

Islanditoxin (200 mg) was hydrolyzed at 130° in a sealed pyrex tube with 20 ml of saturated hot solution of barium hydroxide. After hydrolysis for 20 hours,

---

[10] F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).



α-pyrrole-carboxylic acid (as Nujol mull)
—— synthetic
---- the degradative product, $C_5H_5NO_2$

excess of barium hydroxide was removed from the solution as barium sulfate. The solution was acidified with sulfuric acid and shaken three times with equal volume of ethylacetate. After drying with sodium sulfate, the ethylacetate layer was evaporated to dryness and the residue was subjected to a celullose column chromatography (15 mm × 200 mm) using butyl alcohol saturated with 1.5 N-NH_4OH as a eluting solvent. The Whatman's ashless celullose powder was used. The eluate was collected with the aid of fraction collector in 3 ml fraction each. Cinnamic acid was first eluted and α-pyrrole carboxylic acid was later. The further purification was made by vacuum sublimation.

Cinnamic acid, subliming point 60° in vacuo, m.p. 130°.

yield about 20 mg

Anal. Calcd. for $C_9H_8O_2$: C, 72.96; H, 5.44. Found: C, 72.92; H, 5.47. α-Pyrrole carboxylic acid, subliming at 70° in vacuo, m.p. 180° (decomp.). yield about 15 mg.

Anal. Calcd. for $C_5H_5NO_2$: C, 54.05; H, 4.51; N, 12.61. Found: C, 54.28; H, 4.63; N, 12.72.

Catalytic Reduction of Islanditoxin

A solution of 200 mg of islanditoxin in 20 ml of ethylalcohol was hydrogenated over 50 mg of PtO_2 at 180 atoms. and 130° for 8 hours. After removal of the catalyst by filteration, the solution was evaporated to dryness. The dichloroproline in islanditoxin was reduced about 20% as described later and the extinction of the band at 257 mμ of the reaction mixture indicated that approximately three fourths of β-phenyl-β-amino-propionic acid was hydrogenated. The product, without further separation, was submitted to acid hydrolysis to get proline. The hydrolyzate was subjected to a celullose column chromatography using butyl alcohol, acetic acid and water (4 : 1 : 5) as a solvent system. Although proline did not obtained as the single component but was contaminated with a small amount of serine and α-aminobutyric acid, the main fractions contained proline was collected and were used for optical determination of proline.

Optical Determination of Proline

First, total amount of proline in the sample was measured chemically by the method of F.B. Chivard[11], which was the excellent method for quantitative determinntion of proline among the various amino acids. Next, L-proline content was estimated biologically using *Leuc. mesenteroides*. On the comparison of the two data together, proline obtained catalytic reduction of islanditoxin was found to belong to L-series.

Dehydrochloroislanditoxin (V) and Dehydrochloroislanditoxinic Acid (VI)

Islanditoxin (100 mg) was dissolved in about 30 ml of a mixture of acetone and methanol, and to this solution were added exactly 3 moles of N/10-sodium hydroxide. After standing a few hours, the crystal of dehydrochloroislanditoxin deposited was collected by filteration. yield 20 mg. U.V. max. 268 mμ (ε, 1.5×10^4). From the data of microanalyses, it was better to consider that (V) took methanol rather than water as the solvent of crystallization, which was not lost by vacuum drying at 100°.

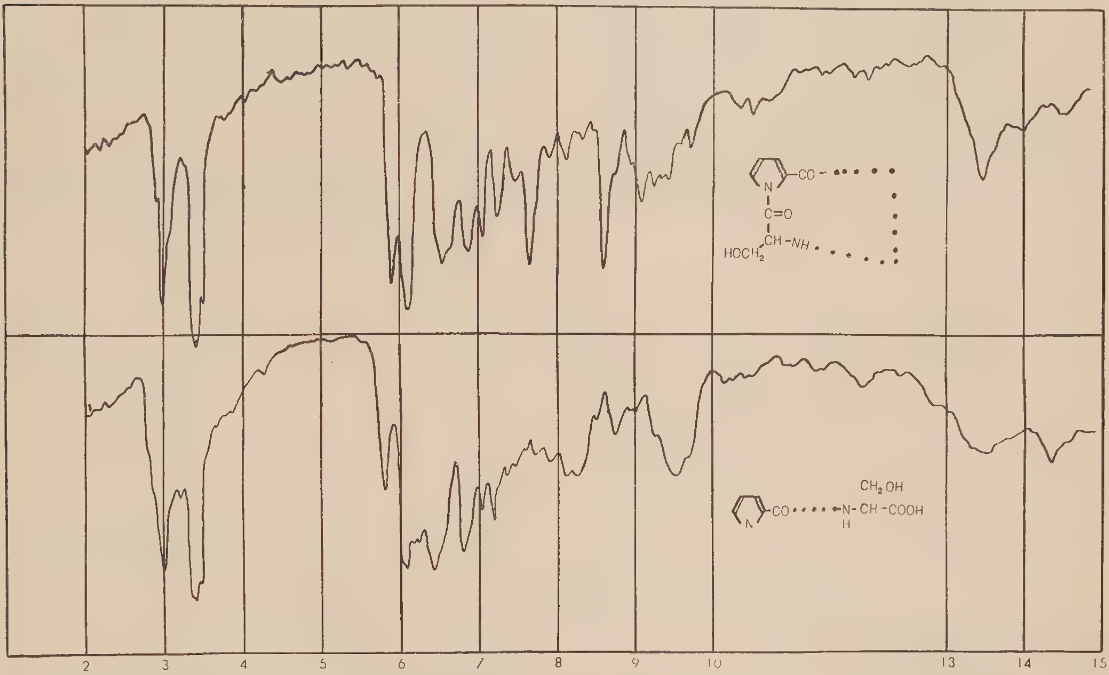
Anal. Calcd. for $C_{24}H_{29}O_7N_5 \cdot CH_3OH$: C, 56.49; H, 6.26; N, 13.18. Found: C, 56.83; H, 6.18; N, 13.24.

---

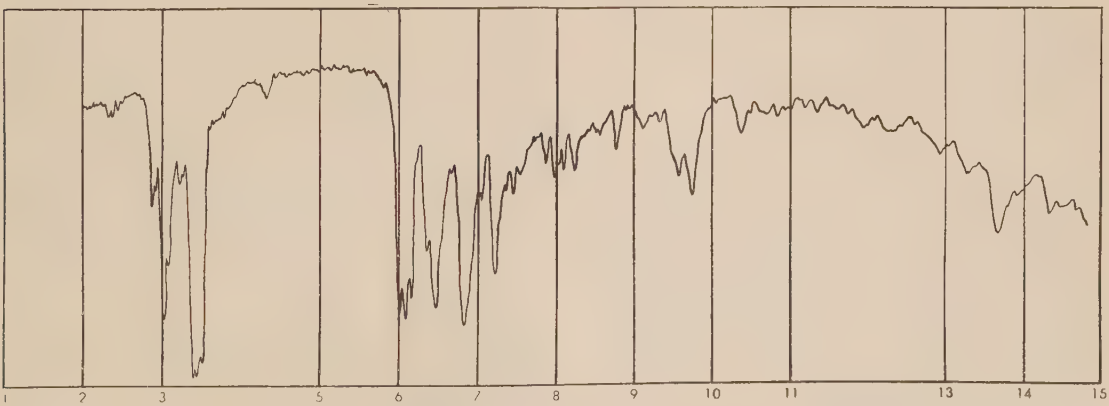
[11] F.B. Chivard, *J.B.C.*, **199**, 91 (1952).

The filterate was evaporated to dryness (pH of solution was almost neutral) and then the residue was subjected to a celullose column chromatography using butyl alcohol saturated with 1.5 N-NH_4OH as a eluting solvent. Aliquot of each eluted tube was tested with the indicator, brom phenol blue, and the fractions contained dehydrochloroislanditoxinic acid were collected. yield 40 mg.

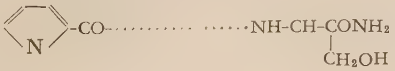
Anal. Calcd. for $C_{24}H_{31}O_8N_5 \cdot H_2O$: C, 53.82; H, 6.21; N, 13.08. Found: C, 53.75; H, 6.44; N, 12.95.

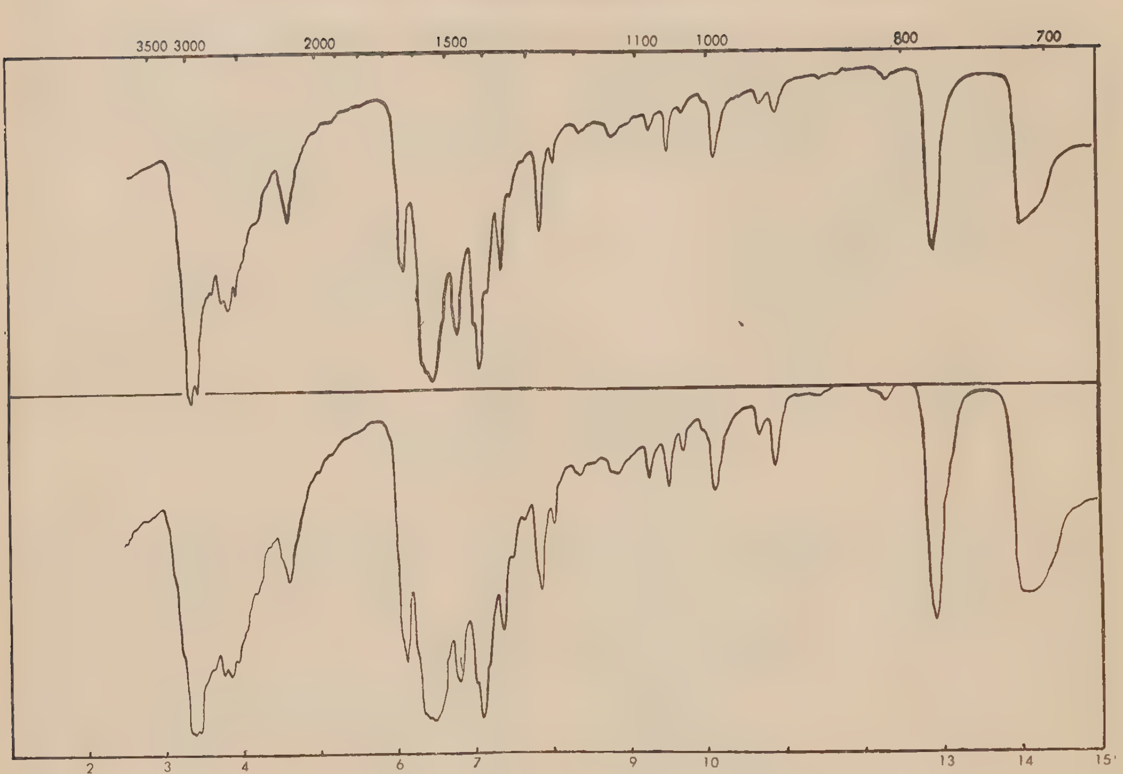


above: dedihydrochloroislanditoxin
below: dedihydrochloroislanditoxinic acid



dedihydrochloroislanditoxinic amide





Above: acid hydrolyzate; $C_9H_{11}NO_2$
Below: synthetic D-β-phenyl-β-amino-propionic acid (as Nujol mull)

Dehydrochloroislanditoxinic Amide

After islanditoxin was added into excess liquid ammonia, the solution was kept at room temperature until ammonia was completely evaporated. The residue was washed with water repeatedly to remove ammonium chlolide, then with acetone and methanol successively and dried at 100°.

m.p. 268~73° (docomp.) U.V. max. 268 mμ (ε, 1.4×10^4)

Anal. Calcd. for $C_{24}H_{32}O_7N_6 \cdot H_2O$: C, 53.92; H, 6.41; N, 15.72. Found: C, 54.13; H, 6.94; N, 15.73.

Quantitative estimation of amide in dehydrochloroislanditoxinic amide was carried out by the method of A.L. Levy et al.[12]. One mole of dehydrochloroislanditoxinic amide liberated 1.3 moles of ammonia in this procedure.

Hydrazinolysis of Peptide

Hydrazinolysis of islanditoxin, peptide A and B and dehydrochloroislanditoxinic acid were carried out according to the method of Akabori[13]. About 5 mg of each of the samples was hydrazinolyzed with 1.5 ml of anhydrous hydrazine at 100° in a sealed tube for 10 hours. The reaction mixture was evaporated in vacuo over sulfuric acid to remove the excess hydrazine. To determine the terminal amino acid, the residue was dissolved in about 2.0 ml of water and subjected to electrophoresis on Toyo filter paper No. 51 in lutidine acetate buffer at pH 6.2 for three hours (20 volt per cm). After electrophoresis, the paper was dried at 80° and again chromatographed during 10 hours in an ascending method for the vertical direction against the moving

Sample	C-terminal amino acid	R_F value*
islanditoxin	none	—
peptide A	α-aminobutyric acid	0.30
peptide B	α-aminobutyric acid	0.30
Dehydrochloroislanditoxinic acid	L-serine	0.13

* As the development solvent, butyl alcohol : acetic acid : water (4 : 1 : 5) was used.

---

[12] A. L. Levy et al., *J. B. C.*, **213**, 187 (1955).

[13] S. Akabori et al., *Bull. Chem. Soc. Japan*, **25**, 214 (1952).

line of the electrophoresis. The the paper was dried, developed with ninhydrin and R_F value of free amino acid was identified. The above results were obtained.

Infrared Spectra of D-β-Phenyl-β-amino Propionic Acid (as Nujol mull)

The infrared spectrum of free amino acid was complicated in the ranges of $1600\sim1400\,\mathrm{cm}^{-1}$ on account of the bands COO^- and benzene. And so, the presence of benzene was presumed from the spectrum of the hydrochloride of the amino acid[4].

Acknowlegements The author wishes to express his sincere thanks to Prof. Y. Sumiki for his guidance throughout this work, to Dr. A. Matsuyama and Dr. K. Miyao in his laboratory for their kind advices and supports to carry out this work. Grateful thanks are also due to Dr. H. Aida of the Institute of Applied Microbiology, University of Tokyo, for the biological estimation of L-proline, to Dr. K. Izaki in his faculty for the optical determination of α- aminobutyric acid.

The cost of this study was defrayed by the Ministry of Agriculture and Forestry, and of Japan Waksman Foundation, to whom author's thanks are due.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 438~442, 1959]

Growth of *Pediococcus soyae* nov. sp. in Highly Concentrated Solutions of Inorganic Salts and Sugars

Studies on the Activities of Bacteria in Soy Sauce Brewing. Part VI

By Kenji SAKAGUCHI

Noda Institute for Scientific Research, Noda City, Chiba Pref.

Received December 11, 1958

Pediococcus soyae nov. sp., which has an inherited salt tolerant nature, is grown in solutions of high osmotic pressure. When this strain is transferred from 0.5% salted medium to a new medium containing 18% sodium chloride, the viable counts of this organism firstly decrease from about one half to one-third of the inoculated cells, and then normal growth occurs. This indicates the occurrence of physiological adaptation at an early stage of growth.

The growth of this lactic acid bacterium is observed in concentrated solutions of various inorganic salts. The solutions containing Na^+, K^+, Cl^-, NO_3^- and SO_4^{--} ions are not toxic for the organism, and the organism can grow in solutions of 133 atm. osmotic pressure, generally. However, Li^+, Ca^{++}, Mg^{++} and Br^+ are, toxic for growth.

In concentrated sugar solutions, this organism also propagates well, and growth is observed in the media containing 50% glucose or 60% sucrose, osmotic pressure being 105 and 84 atm., respectively. Therefore, *Pediococcus soyac* nov. sp. is osmotolerant.

Pediococcus soyae nov. sp.[1~3], which is a characteristic organism in the brewing procedure of Japanese soy sauce, can grow in a media containing a maximum of 24–26% sodium chloride. This salt tolerancy is an inherited characteristic, and is retained even after pedigree culturing of 31st times in 0.5% salted media.[1]

In this paper, the author reports on the phenomenon of physiological adaptation which is observed on the sudden transferring of 0.5% salt containg culture to 18% salted media. This phenomenon has already been described in the osmophilic yeast *Saccharomyces rouxii*[4] and in the cupper tolerant strains of *Saccharomyces ellipsoideus*[5,6].

The growth in concentrated solutions of various inorganic salts and sugars are also examined and some results are presented in this paper.

EXPERIMENTAL

Basal medium. Usually the following basal medium was used, and various salts and sugars were supplemented for each purpose. Heart infusion broth (Difco) 1%, Polypepton (Takeda) 1%, dipotassium phosphate 0.5%, sodium chloride 0.5% and glucose 1%. The hydrogen ion concentration of the media was adjusted to pH 7.0 after autoclaving, because *Ped. soyae* is acid-sensitive and can only grow between the range from pH 5.5 to 9.0[1]. In order to prepare the agar media, 1.3% powdered agar (Takeda), 0.1% sodium thioglycollate and several drops of methylene blue solution (5%) were added to the broth. Sodium thioglycollate maintains the oxido-reduction potentials of the media low enough permitting growth of *Ped. soyae*, and methylene blue serves as the indicator.

Viable counting of *Ped. soyae* grown in 0.5% and 18% salted media.

Ped. soyae strain *d* 2, which had been stocked in 7% salted agar media, was inoculated in 0.5% salted broth and incubated anaerobically at 30°C for four days.

---

[1] K. Sakaguchi, This Bulletin, **22**, 353 (1958).
[2] K. Sakaguchi, *ibid.*, **23**, 22 (1959).
[3] K. Sakaguchi, *ibid.*, **23**, 100 (1959).
[4] H. Onishi, This Bulletin, **21**, 137 (1957).
[5] J. Ashida, *Symposium on Cytological Chemistry*, **1**, 111 (1953)
[6] T. Minagawa, *ibid.*, **1**, 123 (1953).

Five ml of the culture was inoculated to 200 ml in each 0.5 and 18% salted media. They were incubated anaerobically at 30°C, and viable cells grown in the two kinds of broth were counted following the method described below. One ml of the culture was aseptically pipetted and transferred to 50 ml of autoclaved broth, shaked moderately, and 1 ml of the diluted fluid was immediately transferred to the next 50 ml broth. After suitable dilution, 0.5 ml was pipetted, inoculated to 10 ml of melted agar medium and the tube cultured in a high layer. Upon the solidified surface of the agar, was poured uninoculated agar to 2-cm thickness so as to keep the inoculated agar adequately anaerobical. After five to ten day's incubation, colonies grown in the agar were counted.

The culture of 18% salted broth was diluted with the broth of the same salt concentration to avoid osmotic shock, and finally inoculated into two kinds of agar medium containing 0.5% and 18% salt.

RESULTS AND CONCLUSION

Viable cell transition and growth of *Ped. soyae* in 0.5% and 18% salted media.

Ped. soyae has an inherited salt tolerancy, and the upper limit of the salt tolerance is 24–26%. Even in the highly salted media, the fermentation formula of the organism is not changed, proceeding homo-type lactic acid fermentation. The rate of fermentation is however slower[1].

Many drug resistant microorganisms decrease in their viable counts by sudden transfer into the drug added media. When the osmophilic yeast *Saccharomyces rouxii*[4], which is the predominant yeast in soy sauce brewing, is transferred to 18% salted medium from unsalted subculture, only 0.1% to 1% cells gain tolerancy against salt and begin to reproduce, while all the others lose their viability. But the yeast has undoubtedly an inherited salt tolerant nature, and this phenomenon is called physiological adaptation. The cupper tolerance acquistion of *Saccharomyces ellipsoideus*[5,6] is an example of adaptation, and a marked fall of viable cells was noticed during the adaptation phase. *Salmonella pullorum*, *Salmonella schotmülleri* and *Eberthera typhosa*[7] acquired their salt tolerancy through mutation and selection after about one hundred days of incubation. In order to elucidate the adaptation type of *Ped. soyae* by the sudden transfer into highly salted media, the following experiments were conducted.

Ped. soyae strain *d* 2, which had been stock-cultured in 7% salted agar media, was inoculated in a 0.5% salted broth and incubated for four days at 30°C. Five ml of the culture was transferred to two 300 ml conical flasks containing 200 ml of 0.5% salted and 18% salted broth respectively, and incubated at 30°C. The change of viable cell numbers in the two flasks are represented in Fig. 1.

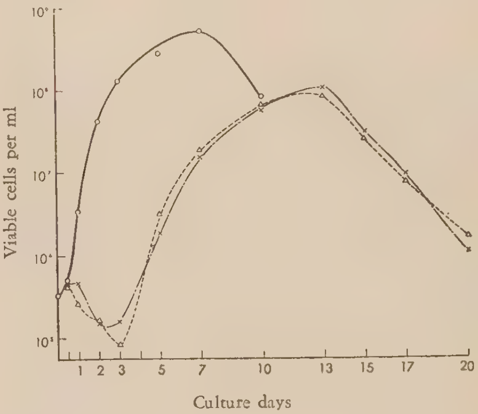


FIG. 1. Growth Curves of *Ped. soyae* in 0.5% and 18% Salted Broth.

—○—	Growth in 0.5% salted broth.
---×---	Growth in 18% salted broth. Colonies counted by 0.5% salted agar.
----△----	Growth in 18% salted broth. Colonies counted by 15% salted agar.

In the 0.5% salted culture, the growth curve of *Ped. soyae* is quite normal, initiating its growth about half a day's lag period, reaching to its maximum counts of 340 million per ml after seven days' incubation, and thereafter the cell numbers decrease rapidly due to the acids produced. However, in the 18% salted broth the viable counts increase in number to about twofold within half a day, thereafter about three-fourth of the cells lose their viability in three days, and then logarithmic growth begins at almost the same speed as usual reaching their maximum counts on the thirteenth day of incubation.

---

[7] J. M. Severens and F. W. Tanner, *J. Bact.*, **49**, 383 (1945).

From these results it is recognizable that in sudden tranferring of *Ped. soyae* from 0.5% salted medium to 18% salted one, the cells show the so-called physiological adaptation as in the case of osmophilic yeast *Saccharomyces rouxii*[4], and reveal the latently inherited salt tolerant character. The ratio of cells which acquire the salt tolerancy is about one half to one-third of the inoculated total cells, and about one-fourth of the one day-cultured cells. This value is much higher compared with *Sacch. rouxii*[4] which decreases its viable counts from 1% to 0.1% of the transferred cells. This phenomenon provides evidence that *Ped. soyae* has an inherited salt tolerant nature.

Fig. 1 also shows that *Ped. soyae* is quite resistant against the osmotic shock of transferring from 18% salted culture to 0.5% salted media. The dotted-curve and the short columned and dotted curve both represent the viable counts of cells in 18% salted culture, but the former counts were counted by the colony numbers grown in 18% salted agar, and the latter in 0.5% salted agar. In any stage of growth, except in the case of the third day, the two curves did not reveal any discrepancy of colony counts. On the contrary, the osmophilic yeasts[4] were very sensitive to osmotic shock, and much decrease in colony counts was observed after the shock.

Growth in media containing a large amount of inorganic salts.

Ped. soyae grows well in media with an extraordinarily high sodium chloride content. The osmotic pressure of the media reaches to 133 atm. at the NaCl concentration of 3 mol (17.6%), and to 187 atm. at the concentration of 4 mol (23.4%)[8] (Table I). The problem whether this

TABLE I. OSMOTIC PRESSURE OF NaCl SOLUTIONS

	Concentration of NaCl	Freezing point depression	Osmotic pressure
1 mol	5.8%	−3.39°C	41.1 atm
2	11.7	−6.93	84.5
3	17.6	−10.89	133.4
4	23.4	−15.11	186.9

TABLE II. GROWTH OF *Ped. soyae* IN MEDIA CONTAINING VARIOUS INORGANIC SALTS

Salts	Osmotic pressure	Lactic acid formed*			
		5 days	8 days	13 days	25 days
NaCl	41.1 atm	1.1 ml	1.6 ml	1.5 ml	1.5 ml
	84.5	1.4	1.4	1.6	1.6
	133.4	0	0	1.4	1.5
	186.9	0	0	0.5	1.5
KCl	41.1	1.0	1.0	1.0	1.0
	84.5	1.0	1.5	1.5	1.4
	133.4	0	1.2	1.5	1.6
	186.9	0	0	0	0
KBr	41.1	1.1	1.1	1.1	1.1
	84.5	0	1.1	1.1	1·1
	133.4	0	0	0	0
	186.9	0	0	0	0
KNO_3	41.1	0.8	1.0	1.0	1.0
	84.5	0.5	1.0	1.2	1.0
	133.4	saturated			
	186.9				
$NaNO_3$	41.1	0.5	0.8	0.8	0.8
	84.5	0.4	0.8	0.8	1.0
	133.4	0	0.5	0.6	1.0
	186.9	0	0	0	0
$MgSO_3$	41.1	0	0	0	0
	84.5	0	0	0	0
	133.4	0	0	0	0
	186.9	0	0	0	0
Na_2SO_4	41.1	1.3	1.4	1.4	1.5
	84.5	1.2	1.6	1.8	2.0
	133.4	0	0	1.7	2.0
	186.9	saturated			
$MgCl_2$	41.1	0	0	0	0
	84.5	0	0	0	0
	133.4	0	0	0	0
	186.9	0	0	0	0
LiCl	41.1	0	0	0	0
	84.5	0	0	0	0
	133.4	0	0	0	0
	186.9	0	0	0	0
NH_4Cl	41.1	0.8	1.0	1.1	1.0
	84.5	—	—	—	—
	133.4	—	—	—	—
	186.9	—	—	—	—

* Values indicate ml of N/20 NaOH consumed in titration of the produced lactic acid in 5 ml culture.

---

[8] Landolt-Börnstein, Physikalich-Chemische Tabellen, **2**, 1452 (1923).

TABLE III. GROWTH OF *Ped. soyae* IN HEAVILY SUGARED MEDIA

	Concentration	Osmotic presure	Titration Value with N/20 NaOH		
			3 days	7 days	10 days
	1%	1.3 atm.	1.40 ml	1.38 ml	1.50 ml
Glucose	20	29.2	1.20	2.25	2.60
	40	74.8	1.40	1.95	2.45
	50	105.0	0.52	1.20	1.45
	60	138.3	0	0	0
	80	204.9	0	0	0
Sucrose	20	19.7	0.75	1.15	1.20
	40	46.7	1.10	1.10	1.25
	50	63.0	0.97	1.37	1.62
	60	84.1	0.44	0.41	0.98
	80	133.5	0	0	0

organism is also tolerant against other inoganic salts or not, namely whether the organism is only salt-tolerant or also osmotolerant, was proposed and the following experiments were carried out. *Ped. soyae.* strain *d* 2 was inoculated in 5 ml of media containing various inorganic salts. The concentrations of these salts were adjusted to the same osmotic pressure with those of media containing 2 mol, 3 mol and 4 mol of sodium chloride, respectively. The inoculated media were incubated anerobically at 30°C and the produced lactic acid was titrated with N/20 sodium hydroxide after five, eight, thirteen and twenty-five days' incubation. The osmotic pressure of various salt solutions were calculated according to Lewis'[4,9] equation of osmotic pressure and freezing-point depression:

$$\Pi = 12.06\Delta - 0.021\Delta^2$$

In this equation, Π and Δ represent osmotic pressure (atm.) and the degree of freezing-point depression (°C), respectively. With concentrated solutions, the calculated value by this equation fits well with the value actually measured[9]. The degree of freezing-point depression of various salt solutions were taken from Landolt-Börnstein's Physikalisch-Chemische Tabellen[8] and Church's data[10].

The results are given in Table II. Na^+, K^+, Cl^-, NO_3^- and SO_4^{--} ions are not toxic for this organism, and growth is observed even under an osmotic pressure of 133 atm. This value coincides with that of 3 mol or a 17.6% sodium chloride solution.

However, Li^+, Mg^+ and Ca^{++} ions are toxic and the media containing these ions did not permit growth at an osmotic pressure of 41 atm.. Br^- ion had a weak toxicity, inhibiting growth at the osmotic pressure of 84.5 atm. by potassium bromide. This toxic effect however appears to be due to the liberated Br_2 molecule and not to Br^- ion. The medium containing about 2 mol of ammonium chloride changed its pH value from 7.0 to 4.5 as a result of incubation at 30°C in the vacuum jar, which completely inhibited the growth of *Ped. soyae.* The organism could grow under osmotic pressure of 187 atm. only in the solution of sodium chloride.

The above-mentioned facts indicate that *Ped. soyae* is not only salt-tolerant, but is also osmotolerant and grows under the circumstance of 133 atm. osmotic pressure provided the toxic inorganic ions are absent. The toxic ions are almost identical with those for osmophilic yeast *Saccharomyces rouxii*[4].

Growth in media containing large amounts of sugars.

Sacch. rouxii, osmophilic yeast in soy mash, grows well in concentrated sugar solutions as well as in concentrated inorganic salt solutions⁴⁾. It is therefore necessary to examine the growth of *Ped. soyae* in sugared media. The sugars tested were glucose and sucrose, the former is a representative fermentable sugar and the latter is also a representative unfermentacle one for this lactic acid bacterium¹⁾. The osmotic pressure of glucose solutions were calculated from the data given in International Critical Tables¹¹⁾, and that of sucrose solutions from the report of Frazer and Myrick¹²⁾.

---

[9] G. N. Lewis, *J. Am. Chem. Soc.*, **30**, 668 (1908).

[10] M. B. Church, *Science*, **74**, 492 (1931).

As shown in Table III, growth of *Ped. soyae* could still be observed in the medium containing 50% glucose, of which osmotic pressure of 105 atm. was attained. In the medium containing a large amount of sucrose, growth was observed in a 60% sucrose solution under 84 atm. osmotic pressure.

From these experiments it is concluded that *Ped. soyae* can grow in sugar solutions of about 100 atm. osmotic pressure, if other nutrient substances are sufficient.

The author wishes to express his hearty thanks to Prof. T. Asai and to Prof. K. Kitahara for their kind guidance and valuable suggestions. He is also indebted to Dr. M. Mogi and Mr. H. Onishi for their valuable advice throughout this work. Thanks are also due to Mr. T. Kuramochi for his earnest and able assistance.

---

11) International Critical Tables, Vol. 4, p. 429 (1928).

12) J.C.W. Frazen and R.T. Myrick, *J. Am. Chem. Soc.*, **38**, 1907 (1916).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 5, p. 443~450, 1959]

P-Factor, a New Peptidic Growth Factor for *Pediococcus soyae*

Studies on the Activities of Bacteria in Soy Sauce Brewing. Part VII

By Kenji Sakaguchi

Noda Iostitute for Scientific Research, Noda City, Chiba Pref.

Received January 14, 1959

The P-factor a new growth factor for *Ped. soyae*, is found in partial acid and enzymic hydrolysates of Hammersten's milk casein, and is therefore, supposed to be a kind of peptide. The well-known peptidic growth promoting substance for *Lact. casei*, strepogenin and its relating substances, are quite ineffective to the author's organism. P-factor exists along with the S-factor which is another growth promoting-factor for this lactic acid bacterium, in soy mash juice and in the water extract of *Aspergillus sojae* mycelium. The former substance is also found in various kinds of commercial peptones. The factor was not substitutable with all of the 19 synthetic peptides tested. S-factor is effective, only in the presence of the P-factor.

In the former report[1], it was shown that *Pediococcus soyae* nov. sp., which is an important lactic acid bacterium for the brewing of Japanese soy sauce[2], grows only very poorly in a complete synthetic medium for bioassay[3,4], and requires two growth factors tentatively named as P- and S-factors. It was also reported that the P-factor is found in enzymic hydrolysates of Hammersten's milk casein and the S-factor in the heart infusion broth (Difco), yeast extract (Difco) and beef extract (Difco). The S-factor was not substiutable with the following substances; glutamine, asparagine, oleic acid, nyacin amide, pyridoxal, vitamin K_3, choline, inositol, yeast nucleic acid, thymine, ornithine, orotic acid, lipoic acid, mevalonic acid, leucovorin, folic acid and vitamin B_{12}.

The present paper deals with the peptidic nature of P-factor, the difference with the well-known peptidic growth promotant strepogenin[5~17], and also the distribution of P-factor in commercially available peptones, soy mash juices and water extracts of *Aspergillus sojae* mycelium.

EXPERIMENTAL

Assay method.

Two and a half ml of the aqueous solutions to be tested for growth promoting activity were placed in test tubes, and an equal-volume of complete synthetic medium for bioassay[3,4] was then added to each of them. The tubes were plugged with cotton, autoclaved at a pressure of 15 pounds for ten minutes, and cooled. Each tube was then added with 1 drop of a saline suspension of *Pediococcus soyae* strain *d* 2, which had been previously grown for four days anaerobically at 30°C in the following medium: yeast extract (Difco) 0.3%, Polypepton

---

1) K. Sakaguchi, This Bulletin, **22**, 353 (1958).

2) K. Sakaguchi, This Bulletin, **23**, 100 (1959).

3) G. Tamura et al, *J. Agr. Chem. Soc. Japan*, **26**, 464, 474 (1952).

4) E. E. Snell, *Nutrition Abstr. & Rev.*, **16**, 497 (1946-47).

5) D. W. Wolley and B. L. Hutchings, *J. Bact.*, **39**, 257 (1940).

6) D. W. Wolley, *J. Exp. Med.*, **73**, 487 (1941).

7) N. Grossowiż, *Proc. Soc. Exp. Biol. & Med.*, **49**, 8 (1942).

8) H. Sprince and D. W. Woolley, *J. Exp., Med.*, **80**, 213 (1944); *J. Am. Chem. Soc.*, **67**, 1734 (1945).

9) D. W. Woolley, *J. Biol. Chem.*, **162**, 383; **164**, 11; **166**, 783 (1946); **171**, 443 (1947); **172**, 71 (1948).

10) M. Womack and W. C. Rose, *J. Biol. Chem.*, **162**, 735 (1946).

11) M. L. Scott and L. C. Norris, *ibid.*, **166**, 481 (1946).

12) L. J. Daniel and M. L. Scott., *ibid.*, **174**, 71 (1948).

13) J. L. Stokes, L. K. Koditschek and T. R. Wood, *ibid.*, **178**, 93 (1949).

14) E. Kodicek and S. P. Mistry, *Biochem. J.*, **173**, 479 (1952)

15) D. W. Woolley and R. B. Merrifield, *J. Am. Chem. Soc.*, **76**, 316 (1954).

16) D. W. Woolley, *Arch. Biochem. Biphys.*, **56**, 265 (1955).

17) R. B. Merrifield and D. W. Woolley, *J. Am. Chem. Soc.*, **78** 358 (1956)

(Takeda) 1.0%, glucose 1.0%, dipotassium phosphate 0.5%, sodium acetate trihydrate 2.0% and pH 7.0. The inoculated tubes were anaerobically incubated at 30°C for fifteen days and the produced lactic acid was periodically titrated with N/20 sodium hydroxide. Consequently, it is necessary to run several separate tests to determine exact growth promoting activity of the sample.

Partial hydrolysis of crystalline beef insulin and Hammersten's milk casein with conc. hydrochloric acid.

Merrifield and Woolley[17] reported that high streptogenin activity is observed in the partial acid hydrolysate of crystalline beef insulin. The same hydrolysate was prepared exactly after the description. Crystalline beef insuline (Shimizu Pharm. Co.) (10 mg) plus 0.25 ml of conc. hydrochloric acid were mixed in a tightly glass-stoppered test tube and immediately placed at 37°C and held at that temperature for twenty hours. The hydrochloric acid was removed as completely as possible by distillation under reduced pressure in a water-bath at 37°C. The residue was dried in vacuo over KOH for eighteen hours, dissolved in 1 ml of water, adjusted to pH 7 with NH_4OH.

Hammersten's milk casein (Merck) was also hydrolysed in the same manner. One hundred mg of casein was added with 2.5 ml of conc. HCl and held at 37°C for twenty hours, distilled, dissolved in 10 ml of water and neutralized.

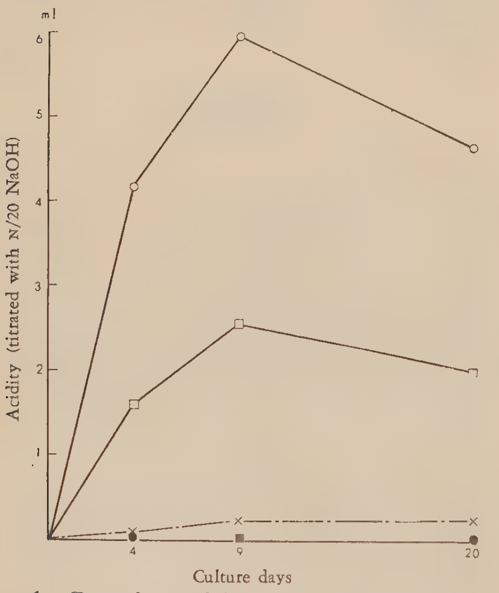


FIG. 1. Dependency of Action of S-factor on P-factor

—●— Synthetic medium
—□— Supplemented with Polypepton 1.0%
---×--- Supplemented with beef extract 0.3%
—○— Supplemented with both Polypepton 1.0% and beef extract 0.3%

RESULTS AND DISCUSSION

Dependency of the action of S-factor on P-factor. Approximately a half of maximum growth of *Ped. soyae* is observed when the basal medium is fortified with P-factor as a partial hydrolysate of milk casein. Further addition of S-factor as Difco beef extract, heart infusion broth or yeast extract results in full growth of the organism[1]. It is questionable however, whether the mere addition of S-factor has any effect on

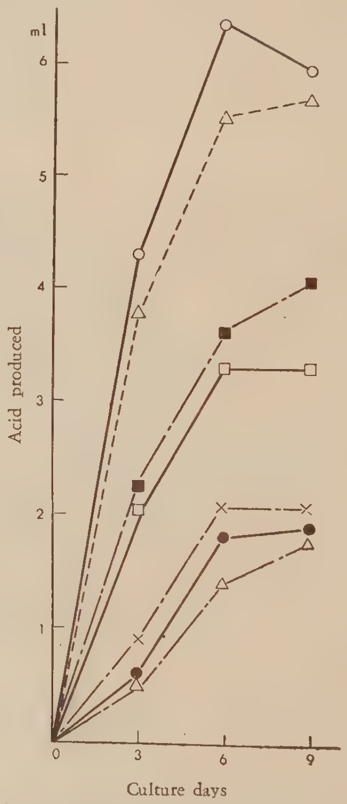


FIG. 2. Effective Concentration of Polypepton

—●— Synthetic medium
—○— Supplemented with heart infusion broth 1.0% and Polypepton 1.0%.
---△--- Suppl. with Yeast extract 0.3% and Polypepton 1.0%.
—■— Suppl. with Polypepton 2.0%.
—□— 1.0%
---×--- 0.1%.
---△--- 0.01%.

stimulation of growth of *Ped. soyae*. Therefore, Difco's beef extract which was seemed to be a rich source of S-factor but not of the peptidic P-factor, was added up to 0.3% in the complete synthetic medium for bioassay[1,3,4], and growth curves were determined as shown in Fig. 1. From the curves it is recognized that the addition of both S-factor and P-factor stimulated the growth of *Ped. soyae* strain *d* 2, but the addition of the S-factor alone was almost ineffective for its growth. Therefore, S-factor was effective only in the presence of P-factor.

Effective concentration of Polypeton. The minimum effective concentration of "Polypepton" (Takeda) was examined by adding the substance to concentrations of 2%, 1%, 0.1% and 0.01% into the synthetic medium. The growth curves of *Ped. soyae* strain *d* 2 on these media is shown in Fig. 2. The figure shows that significant growth is not observed below the Polypepton concentration of 0.1%, and a concentration of 1.0% is necessary for full effect of the P-factor.

The figure also indicates the fact that the S-factor can not be substituted with a high concentration of P-factor. Even in the medium which contained 2% Polypepton much less growth was observed as compared with that in the medium which contained both P- and S-factors, namely, supplemented with heart infusion broth (Difco) 1.0% or yeast extract (Difco) 0.3% plus Polypepton (Takeda) 1.0%. Therefore, both the P-factor and S-factor are different substances.

Meanwhile it is worthy to note that a small but measurable growth of the organism was observable in the synthetic medium at this stage (Compare Fig. 2 with Fig. 1). This probably depends on the inoculum size of the organism partly, and also, perhaps on the long stocking of the synthetic medium prepared for two weeks in the refrigerator, which is known to cause some unknown change between antagonistic amino acids[18].

Existence of both P-factor and S-factor in the extract of *Asp. sojae* mold bran and in the unpasteurized soy sauce. To ascertain the existence of the two substances in soy sauce and in soy Koji, *Ped. soyae d* 2 was grown in the synthetic medium supplemented with the unpasteurized soy sauce and with the water extract of molded bran grown with *Aspergillus sojae* strain KS., the soy koji mold[2,19] in use at Noda Shoyu factories. A half-ml of soy sauce which was not yet pasteurized and not added with the antifungal agent (butyl-*p*-hydroxy-benzoate) was added to 4.5 ml of the synthetic medium. The content of the supplemented nitrogenous compounds in the medium was about 1% as protein. The medium supplemented with the water extract of molded bran was prepared as follows: 10 g of molded bran purely cultured with *Asp. sojae* was extracted with 40 ml of distilled water for four hours in the refrigerator, filtered, and 0.5 ml of the filtrate was added to 4.5 ml of the synthetic medium.

As shown in Fig. 3, both the P-factor and S-factor exist in the water extract of molded bran and in the unpasteurized soy sauce. However, it was observed that *Ped. soyae* could not easily grow in soy sauce supplemented with glucose and the pH adjusted at 7.0. This is probably due to many growth inhibitors produced in soy sauce during a maturing[20] period of one year.

Existence of P-factor in several commercial petones. P-factor, which is a peptidic substance produced by an enzymic partial hydrolysis of milk casein, actually exists in "Polypepton" of Takeda Pharmaceutical Co. Ltd.. The existence of this factor in other commercially available peptones is recognized in this experiment. Bacto-peptone, Bacto-tryptone (Difco) or Polypepton (Takeda) were dissolved to 1.0% in the synthetic medium, and growth of *Ped. soyae d* 2 was examined (Fig. 4). P-factor also exists in Difco's two peptones and higher growth responces than in the medium supplemented with Takeda's Polypepton were observed. The reason why higher

---

[18] M.M. Yragui, et al., *J. Bact.*, **69**, 20 (1955).

[19] K. Sakaguchi, This Bulletin, **22**, 345 (1958).

[20] T. Yokotsuka, *J. Agr. Chem. Soc. Japan*, **23**, 200 (1948); **24**, 355, 358, 402, 407 (1951); **25**, 206, 401, 446 (1952): **27**, 276 334, 359, 549 (1953).

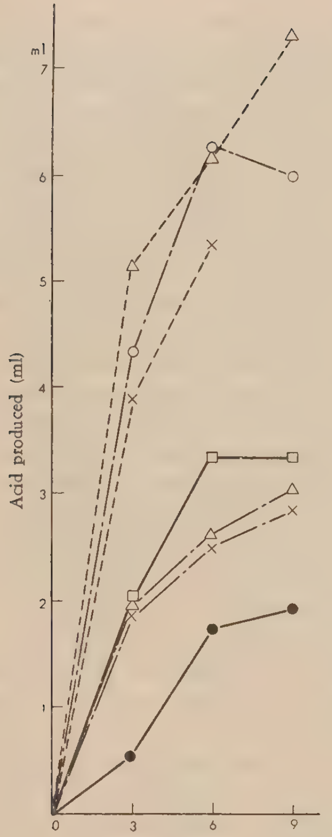


FIG. 3. Existence of P-Factor and S-Factor in Unpasteurized Soy Sauce and in Water Extract of *Aspergillus sojae* Mycelium.

——●—— Synthetic medium.
——□—— Supplemented with Polypepton 1.0%.
—·—○—·— Suppl. with heart infusion broth 1.0% and Polypepton 1.0%.
----△---- Suppl. with soy sauce (1.0 % as protein)
—·—△—·— (0.1 % as protein)
----×---- Suppl. with the extract of *Aspergillus* mycelium (1 : 4, 0.5 ml).
—·—×—·— (1 : 4, 0.05 ml).

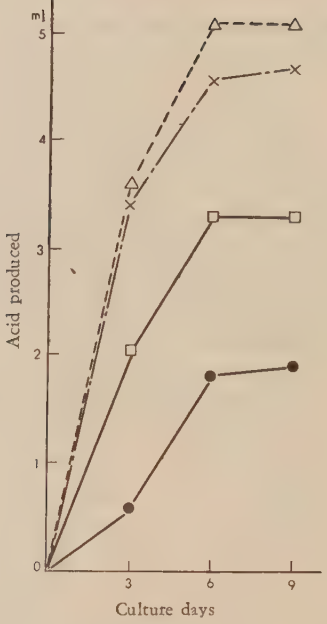


FIG. 4. Existence of P-Factor in Various Commercial Peptones.

——●—— Synthetic medium.
——□—— Supplemented with Polypepton (Takeda).
----△---- Suppl. with Bacto-peptone (Difco).
—·—×—·— Suppl. with Bacto-tryptone (Difco).

growths are observed on Difco's two peptones will probably be explained by contamination of the S-factor.

Tolerancy of P-factor against attacks of proteinases or HCl. Since the P-factor is assumed to be a kind of peptide, it is conceivable that this substance will lose its biological activity through an attack by various proteinases or hydrochloric acid. Polypepton was hydrolysed with HCl and with various proteinases in the following way and their activities on *Ped. soyae* were examined. The proteinases used were; pepsin (Twice crystallized, GBI.), trypsin (Crystalline, GBI.), Nagarse (A crystalline *Bacillus subtilis* proteinase. Nagase Ind. Co.), *Asp. sojae* alkaline proteinase[21,22] (Crystalline, generously supplied by Mr. D. Fukushima of our Institute), *Aspergillus saitoi* acid proteinase[23] (Crystalline, kindly supplied by Dr. F. Yoshida and Mr. M. Nagasawa of our Institute) and *Streptomyces* protease[24,25] G (Crystalline, kindly supplied by Mr. M. Nomoto of the Scientific Research Institute). Among these proteinases, *Streptomyces* protease G which

---

[21] T. Mizunuma and N. Iguchi, This Bulletin, **22**, 35 (1958).
[22] W. G. Crewther and F. G. Lennox, *Aust. J. Biol. Sci.*, **6**, 428 (1953).
[23] F. Yoshida and M. Nagasawa, This Bulletin, **20**, 252, 257, 262 (1956).
[24] M. Nomoto, et al., *Symposia on Enzyme Chemistry* (Japan), **12**, 24 (1957).
[25] M. Nomoto, et al., *J. Biochem.*, in press.

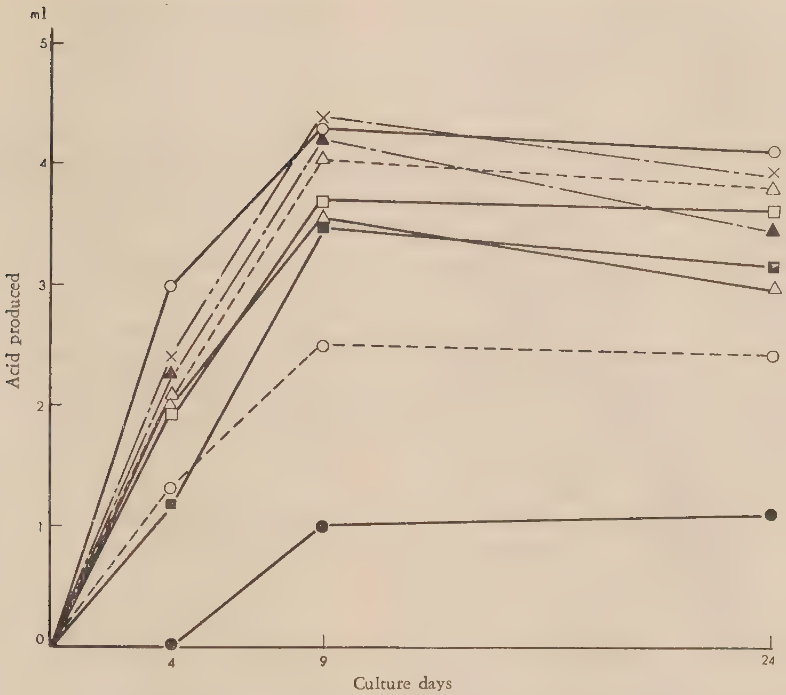


FIG. 5. Digestion of P-Factor with Various Proteinases and with HCl.

——●—— Synthetic medium.
——○—— Supplemented with unattacked Polypepton.
----△---- Attacked by pepsin.
-—-×-—- Attacked by trypsin.
----○---- Attacked by *Aspergillus sojae* alkaline proteinase.
——■—— Attacked by *Aspergillus saitoi* acid proteinase.
——□—— Attacked by *Bacillus subtilis* proteinase.
-—-▲-—- Attacked by *Streptomyces* protease G.

is produced in the culture filtrate of *Str. griseus* has the ability to degrade protein molecule more completely than trypsin. This preteinase can digest milk casein perfectly so that under the best conditions, almost 80 to 90% of the constituent amino acids become free. In buffer solutions having the optimal pH value of every enzyme, Polypepton was dissolved to 10% concentration, the enzymes were added at the concentration of 0.05%, and incubated at 37°C for four and a half hours. After incubation, the reaction was stopped by keeping the mixture solutions in a boiling water bath for five minutes and thereafter stored in a refrigerator. The procedure of decomposing Polypepton partially with hydrochloric acid was exactly the same as the method employed in the preparation of strepogenin rich hydrolysate from insulin, which has already been described in this report.

Results are shown in Fig. 5. The P-factor, which is included in Polypepton, has strong resistance against the attack of many proteolytic enzymes and also of cold hydrochloric acid. Its growth stimulation activity for *Ped. soyae* is not diminished after such treatments. Only *Aspergillus sojae* alkaline proteinase succeeded in reducing activity to about half. Especially, the P-factor, was quite tolerant to the attack of trypsin and no decrease of activity was observed after treatment. This is of no wonder, because Polypeton is produced by hydrolysing milk casein exhaustively with an enzyme preparation of which the main component is trypsin.

P-factor activity of various partially hydrolysed

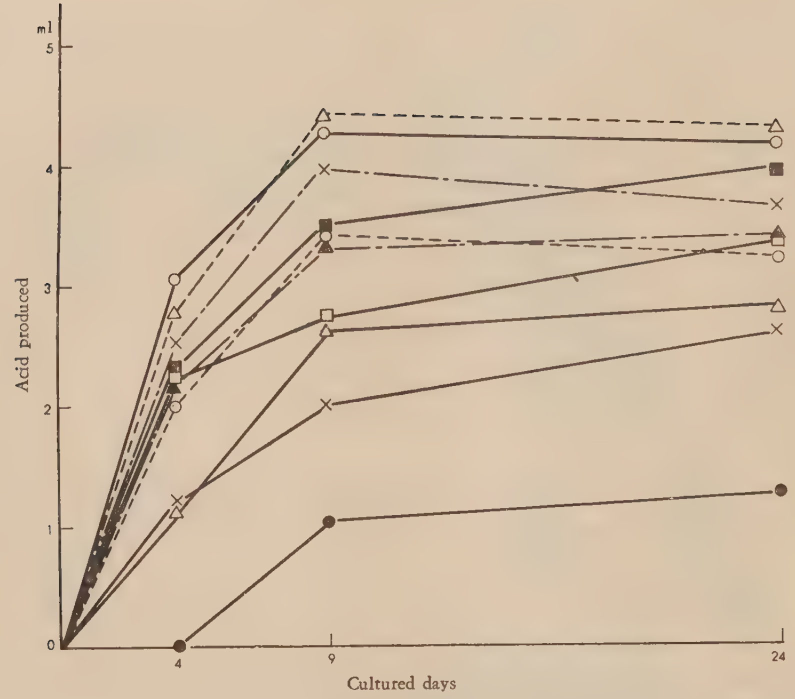


FIG. 6. P-Factor Activity Represented in Various Hydrolysates of Milk Casein.

——●—— Synthetic medium.
——○—— Supplemented with Polypepton.
——×—— Suppl. with unhydrolysed casein.
----△---- Suppl. with hydrolysate by HCl.
——△—— Suppl. with hydrol. by pepsin.
—·—×—·— Suppl. with hydrol. by trypsin.
······○······ Suppl. with hydrol. by *Aspergillus sojae* alkaline proteinase.
——■—— Suppl. with hydrol. by *Aspergillus saitoi* acid proteinase.
——□—— Suppl. with hydrol. by *Bacillus subtilis* proteinase.
—·—▲—·— Suppl. with hydrol. by *Streptomyces* protease G.

milk casein. Fig. 6 shows the growth curves of *Ped. soyae d* 2 which have been inoculated in the synthetic medium supplemented with various enzymic and acid partial hydrolysates of Hammersten's milk casein.

The enzymes and the methods of hydrolysis were identical with those employed in the former experiment. The hydrolysates were added to the synthetic medium at the concentration of 1.0% as protein.

From this figure it is shown that the various enzymic partial hydrolysates of milk casein retain more or less P-factor activity, and also, unhydrolysed whole milk casein somewhat stimulates the growth of the organism. However, the most effective hydrolysate for the lactic acid bacteria is found among those hydrolysates including even Polypepton itself, the one hydrolysed by conc. hydrochloric acid at 37°C for twenty hours. This fact seems to provide evidence that the P-factor is a kind of peptide constituted of the protein molecule of milk casein.

Effect of various synthetic peptides. From the studies on *Leuconostoc mesenteroides*26) and on other microorganisms it is known that small peptides are generally more nutritious to them than the mere addition of the constitutent amino

---

26) A. Miller, A. Neidle and H. Waelsch, *Arch. Biochem. Biophys.*, **56**, 11 (1955).

acids. Therefore, it might be said that the peptidic P-factor is not a specific peptide (it may be a group of peptides), but many peptidic molecules are all effective for *Ped. soyae.*

In order to exclude this possibility, various synthetic peptides were collected and their activites were measured by adding them a concentration of 500 γ per ml of the synthetic medium. The collected syntetic peptides were; L-prolyl-L-valine, L-leucyl-L-proline, their anhydrides which are reported by Koaze to be the germination promotants for plant seeds[27], glutathione, glycylglycine, glycyl-L-leucine, L-leucylglycine, L-leucine amide, L-leucyl-glycyl-glycine, L-alanyl-glycyl-glycine, DL-alanyl-DL-aspartic acid, carbobenzoxy-DL-phenylalanine, carbobenzoxy-L-tyrosine, carbobenzoxy-L-glutamyl-L-tyrosine, α-benzoyl-DL-arginine-ethyl-ester, α-benzoyl-DL-arginine-amide, α-benzoyl-glycine-amide and L-chloro-acetyl-L-tyrosine.

As shown in Table I, none of these peptides could promote the growth of *Ped. soyae.* Since it is reported that glutathione has a weak strepogenin activity for *Lact. casei,* this substance was examined at a concentration as high as 1.0 mg/ml, but no effect was observed. These results show that P-factor activity is not due to unspecific peptides, but is due to a group of specific peptides which are biologically active.

TABLE I. NUTRITIONAL EFFECT OF SYNTHETIC PEPTIDES ON *Ped. soyae*

Peptides	Acid produced*
L-Pro-L-Val anhydride	0 ml
L-Leu-L-Pro anhydride	0
L-Pro-L-Val	0
L-Pro-L-Leu	0
Gly-Gly	0
Gly-L-Leu	0
L-Leu-NH_2	0
L-Leu-Gly	0
L-Leu-Gly-Gly	0
DL-Ala-DL-Asp	0
DL-Ala-Gly-Gly	0
carbobenzoxy-DL-Ala	0
Cb-DL-Phe	0
Cb-L-Tyr	0
Cb-L-Glu-L-Tyr	0
α-benzoyl-L-Arg-ethylester	0
benzoyl-L-Arg-NH_2	0
benzoyl-Gly-NH_2	0
L-chloroacetyl-L-Tyr	0
glutathione (1 mg/ml)	0

* After 8 days incubation, 5 ml of the cultures were titrated with N/20 NaOH.

Difference of P-factor from strepogenin. Strepogenin is well known[5~17] as for the peptidic growth promotant for lactic acid bacteria. The substance, which stimulates the growth of haemolytic *Streptococci*, *Lactobacillus casei* and Smith's *Streptococcus lactis*, is included in acidic and enzymic partial hydrolysates of various protein, especially insulin and milk casein. It is not a single peptide, and the identified several active peptides are all different in their molecular size and their activities[9,15,17]. Among these the most effective are: the pentapeptide seryl-histydyl-leucyl-valyl-glutamic acid and the heptapeptide seryl-histydyl-leucyl-valyl-glutamyl-alanyl-leucine which are isolated from the acid hydrolysate of insuline[17]. The pentapeptide was synthesized by Prof. Akabori and his collaborators at the University of Osaka, and this substance (100 γ/ml) was tested for the P-factor activity. Two peptidic hormones of the posterior lobe of pituitary body, vasopressin[28] and oxytocin[29], are known to have high potencies of strepogenin activity[15], and these substances were also tested. Two International Units of synthesized oxytocin (Sandoz Co. Ltd.. Labelled as Syntocinon.) were added to 5 ml of the synthetic medium for lactic acid bacteria, autoclaved at 115°C for ten minutes, and inoculated with the washed supension of *Ped. soyae d* 2. The vasopressin preparation used here is an injection of Parke, Davis & Co., named Pitressin, which is not a synthetic drug, but a fractional extract of the posterior pituitary gland, and can not be considered to be a pure substance. Moreover, it was necessary to remove the added preservative chlorobutanol by extraction with

---

27) Y. Koaze, This Bulletin, **21**, 197 (1957); **22**, 91, 98, 238 (1958).

28) V. du Vigneaud, et al., *J. Am. Chem. Soc.*, **75**, 4880 (1953).

29) V. du Vigneaud, et al., *ibid.*, **75**, 4879 (1953).

ether. After pretreatment, 4 International Units of the subtance were examined as usual. The partial acid hydrolysate of insulin is reported by Merrifield and Wolley[17) to be rich of strepogenin activity, and 10 mg of the hydrolysate was added into the synthetic medium.

TABLE II. NUTRITIONAL EFFECT OF STREPOGENIN AND ITS RELATING SUBSTNCES ON *Ped. soyae*

Peptides	Concentration	Acid produced
Ser-His-Leu-Val-Glu	100 γ/ml	0 ml
partial hydrolysate of insulin	2 mg/ml	0
vasopressin	4 units/ml	0
oxytocin	0.4 units/ml	0

Table II indicates that all strepogenin and its relating substances are ineffective to stimulate the growth of *Ped. soyae*. It is thus affirmable that P-factor is a series of biologically active peptidic substances different from strepogenin*.

The author wishes to express his hearty thanks to Prof. T. Asai and Prof. K. Kitahara of the Institute of Applied Microbiology, Univerty of Tokyo for their kind guidance. Thanks are also due to Dr. G. Tamura, Dr. A. Obayashi, Mr. Y. Koaze of the University of Tokyo and Mr. J. Kirimura of the Sericulture Experiment Station for their generous supply of precious growth promotants. The author is greatly indebted to Dr. M. Mogi, and members of this Institute for their kind advice and to Mr. T. Kuramochi for his willing and able technical assistance.

---

* Recently, this conclusion was affirmed by D. W. Woolley in his private letter.

Note

Biological Conversion of Shikimic Acid or Quinic Acid to Protocatechuic Acid, Gallic Acid and Aromatic Amino Acids

Sir:

The authors have found that several *Bacillus* are able to utilize the ammonium salts of shikimic acid (SA), quinic acid (QA), 5-dehydroshikimic acid (DHS) and 5-dehydroquinic acid (DHQ) as their sources of carbon and nitrogen[1]. One of these micro-organisms isolated by the authors is *Bacillus cereus*, which forms protocatechuic acid (PCA), gallic acid (GA), phenyl alanine and tyrosine from SA or QA.

This *Bacillus* was cultured in 10-ml portion of a nutrient medium in the dark at 30°. The medium was prepared as follows: 0.2 g of SA or 1 g of QA, 0.3 g of $MgSO_4 \cdot 7H_2O$ and 0.1 g of KH_2PO_4 were dissolved with tap water to 100 ml and the pH was adjusted to 7.2 with a small amount of ammonia water.

After 13 days incubation, the ether extract of an acidified medium was found to contain PCA and GA after application of paper chromatography employing two solvent systems (butanol-acetic acid-water, 4 : 1 : 2 V/V and 80% phenol were used as the developing solvent and ferric chloride solution as spray reagent) and, these compounds were also identified by the methods reported previously[1].

The aqueous phase of the medium revealed four spots as shown by the bromocresol green indicator on paper chromatograms. These acids were identified by comporison of R_F values with these values obtained from authentic samples of the respective acids and found to be SA, QA, DHS and DHQ. On the other hand, aromatic amino acids in the aqueous solution were isolated by the method of activated carbon adsorption[2], and identified by paper chromatography (the upper phase of the mixture of butanol, acetic acid and water (4 : 1 : 5 V/V) was used as the solvent).

At intervals of three days after inoculation, the cells of *Bacillus* were removed from the medium by filteration, and the acidified filtrate and washings were extracted with ether. The ether was distilled off, and the residue was dissolved in a small amount (1 ml) of ethanol. The yellowish solution produced was immediately developed in a system of butanol, acetic acid and water (4 : 1 : 2 V/V), both PCA and GA were determined colorimetrically with Beckman spectrometer: the amount of the former was directly estimated through the optical densities at 260 and 295 mμ in the ethanol extract of paper strip of its chromatogram, and the amount of the latter from density at 275 mμ in the ethanal extract.

The aqueous layer of ether extraction was concentrated in vacuo to syrup at 60°, dissolved in a small amount of ethanol. The ethanol-insoluble part was added in 10 ml of water, filtrated and aromatic amino acids in the filtrate were isolated by above method. In the paper chromatography of these metabolic products, the solvent system was the mixture of butanol, acetic acid and water (4 : 1 : 5 V/V). The amounts of SA, QA, DHS and DHQ was determined by the methods of Hattori[3] and Nagasawa[4], while that of phenyl alanine and tyrosine by Trolle's method[5].

Yields of the metabolic products are given in Figures 1 and 2. When *Bacillus cereus* was in-

---

1) H. Ishikawa and T. Oki, *J. Agr. Chem. Soc. Japan*, **33**, 319 (1959).

2) S. M. Partridge, *Biochem. J.*, **44**, 521 (1949).

3) S. Hattori, S. Yoshida and M. Hasegawa, *Arch. Biochem. and Biophys.*, **74**, 480 (1958).

4) M. Nagasawa, This Bulletin, **22**, 205 (1958).

5) W. Troll and S. Cannan, *J. Biol. Chem.*, **200**, 803 (1953).

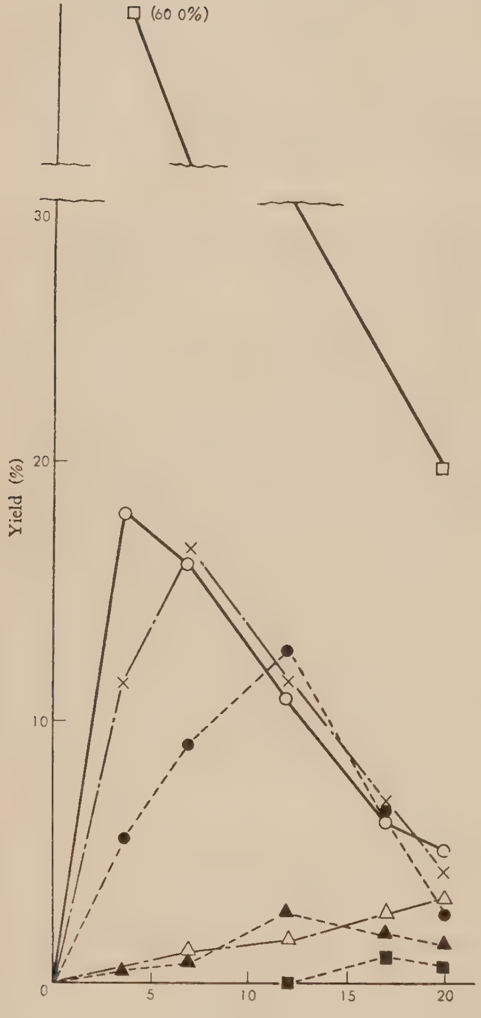


FIG. 1. Formation of the Main Metabolic Products from Shikimic Acid by *Bacillus cereus* in Medium at 30°.

□——□ : Shikimic acid
○——○ : Quinic acid
×-·-× : 5-Dehydroshikimic acid
●---● : Protocatechuic acid
■-··-■ : Gallic acid
△---△ : Phenyl alanine
▲-·-▲ : Tyrosine

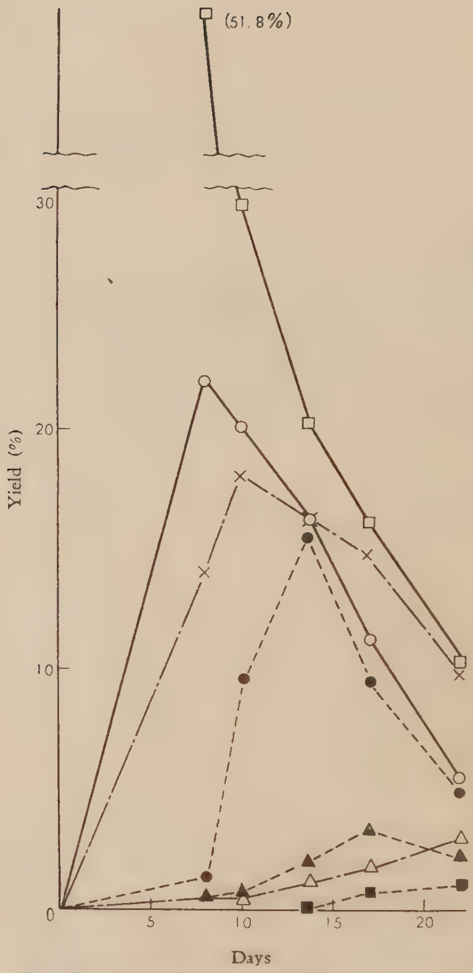


FIG. 2. Formation of the Main Metabolic Products from Quinic acid by *Bacillus cereus* in Medium at 30°.

○——○ : Shikimic acid
□——□ : Quinic acid
×-·-× : 5-Dehydroshikimic acid
●---● : Protocatechuic acid
■-··-■ : Gallic acid
△---△ : Phenyl alanin
▲-·-▲ : Tyrosine

cubated for 13 days on the medium containing SA, the yield of PCA reached its maximum and about two-thirds of SA consumed were converted to DHS (12% yield), QA (11% yield), PCA (13% yield), GA (0.3% yield), phenyl alanine (2% yield) and tyrosine (2.5% yield). Despite this the amount of DHQ was extremely small. This *Bacillus* incubated for 10 days in an aqueous medium with 0.2% DHS yielded a large amount (20% yield) of PCA with a small amount of SA, QA, GA and aromatic amino

TABLE I. THE RELATIVE VEROSITY OF THE FORMATION OF PROTOCATECHUIC ACID, GALLIC ACID AND AROMATIC AMINO ACIDS AS FUNCTION OF pH

pH	Protocatechuic acid	Gallic acid	Phenyl alanine	Tyrosine
5.2	0.12 mg	0.00 mg	0.22 mg	0.23 mg
6.0	0.22	0.18	0.29	0.23
7.0	0.82	0.23	0.32	0.24
7.5	5.00	0.55	0.35	0.25
7.8	5.37	0.51	0.33	0.24
8.3	5.02	0.45	0.32	0.24

The concentration of shikimic acid in medium: 10 mg/10 ml.
The time of incubation: 13 days.

acids. It was also observed that, under the above condition, the biogenesis of DHS began earlier than the formation of these aromatic compounds.

Although residual DHS was usually observed after prolonged incubation, the conversion of DHS to PCA was essentially irreversible, i.e. when this *Bacillus* developed for 5 or 10 days on the medium composed of a small amount (0.2%) of PCA, GA, phenyl alanine or tyrosine, QA SA, DHS and DHQ were not produced. The biological conversion of PCA to GA was however confirmed to be carried out on the PCA-containing medium.

Figure 2 shows a relationship between the yield of the metabolic products of QA as the sole source of carbon at each stage of development of *Bacillus cereus*.

From these results, it was ascertained that the main metabolic products of SA and QA are DHS, PCA, GA, phenyl alanine and tyrosine.

The relative velocities of PCA-formation as function of pH are shown in Table I. The pH maximum is between 7.5 and 7.8. The biogenesis of PCA and GA from SA, QA or DHS was completely inhibited by *p*-chloromercuric benzoate (2×10^{-3} M), $CuSO_4\cdot5H_2O$ (1×10^{-3} M) or $Hg(CH_3COO)_2$ (1×10^{-3} M) which were added simultaneously with incubation, while the formation of aromatic amino acids was only partly inhibited.

Neurospora and *Pseudomonas* are known to produce PCA, from the studies of Tatum[6], Gross[7] and Hattori et al[3]. and, when these micro-organisms were grown on SA as the sole source of carbon, it was believed that DHS is a precursor of PCA. Furthermore, the consideration that DHS assumes the role of an intermediate in the biogenesis of aromatic amino acids was introduced by Davis[8].

The result described above indicates that DHS is an important intermediate in the formation of PCA, GA and aromatic amino acids from SA or QA by *Bacillus* and that GA is prepared from PCA by biological hydroxylation.

If PCA and GA in the medium of *Bacillus cereus* are formed through a similar pathway to that of the plant, the results of the present experiments may serve to provide an important suggestion in theorizing the formation of PCA, GA and gallo-tannin in plants.

Hisao ISHIKAWA
Tae OKI

Faculty of Agriculture,
Ehime University,
Matsuyama, Japan.

Received Feburay 27, 1959.

---

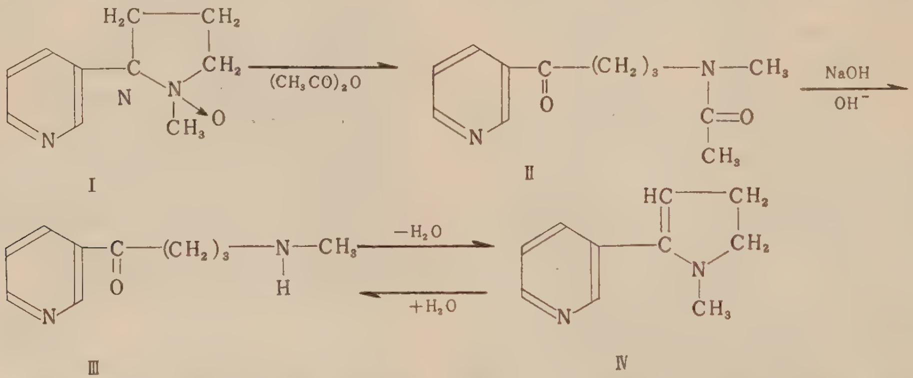
6) E. L. Tatum, S. R. Gross, G. Ehrensvärd and L. Garnjobst, *Proc. Natl. Acad. Sci. U. S.*, **40**, 271 (1954).
7) S. R. Gross, *J. Biol. Chem.*, **233**, 1146 (1958).
8) B. D. Gavis, *Advances in Enzymol.*, **16**, 247 (1955).

A New Synthesis of N-Methylmyosmine

Sir:

From several transformation studies on nicotine[1,2,3], it has been shown that N-methylmyosmine is an important intermediate in the degradation of this alkaloid.

Pseudoöxynicotine (III) was first described by Pinner and Wolfenstein[4]. More recently, Haines and Eisner[5] have re-examined the earlier work and proved the compound (III) to be an open chain hydrolytic product of N-methylmyosmine (IV). By exactly following the previous method, the latter workers heated oxynicotine (I) in a sealed tube with hydrochloric acid at 140° for several hours and obtained dihydrochloride of N-methylmyosmine (IV) in a yield of 12%.



The authors have found a new synthetic method for compound (III) which also started with oxynicotine (I) under a very much milder condition and gave a yield of 60～70%. In this study, oxynicotine (I), formerly known only as a hygroscopic viscous oil, was also obtained in a white crystalline form, m.p. 154–155.2°, $[\alpha]_D^{21.5}=+65.3$ (c=5.13 methanol), refined on an almina column and recrystallyzed from ethyl acetate.

Oxynicotine (31 g.) was treated with acetic anhydride (25 ml.) in an ice-bath; the reaction temperature was maintained below 40°. Another 25 ml. of acetic anhydride was added and the mixture was refluxed at 70° for half an hour. The mixture was made alkaline and extracted with chloroform three times and the combined chloroform extracts were concentrated by distillation, to yield a dark red oil. On fractional distillation, the oil yielded a main fraction boiling at 198–202°/3.0 mm. which was shown to be 1-(3′-pyridyl)-3-(N-acethylmethylamino)-1-propanone (II); yield, 22.15 g., $n_D^{20}=1.5397$.

Anal. Calcd. for $C_{12}H_{16}N_2O_2$: C, 65.43; H, 7.32; N, 12.72. Found: C, 64.94; H, 7.10; N, 12.63.

The infrared spectrum of the compound exhibited two strong absorptions at 1680 and 1630 cm^{-1}. The ultraviolet absorption spectra of the compound showed λ_{max} 268 mμ ($\varepsilon=3000$), λ_{max} 230 mμ ($\varepsilon=7400$) and λ_{min} 252 mμ ($\varepsilon=2360$)

---

1) E. Wada and K. Yamasaki, *J. Am. Chem. Soc.*, **76**, 155 (1954).

2) E. Wada, T. Kisaki and K. Saito, *Arch. Biochem. Biophys.*, **79**, 124 (1959).

3) W. G. Frankenburg, A. G. Gottscho, A. A. Vaitekunas and R. M. Zacharius, *J. Am. Chem. Soc.*, **77**, 5730 (1955).

4) A. Pinner and R. Wolfenstein, *Ber.*, **25**, 1428 (1892).

5) P. G. Haines and A. Eisner, *J. Am. Chem. Soc.*, **72**, 1719 (1950).

in aqueous solution and λ_{max} 263 mμ (ε=4360) and λ_{min} 241 mμ (ε=1620) in 0.03 N HCl aqueous solution.

The N-acethylamino ketone (II) (1.0 g.) in 12 ml. of ethanol was refluxed with 2,4-dinitrophenylhydrazine (1.0 g.) and 4 ml. of conc. hydrochloric acid. The reaction mixture was kept in the refrigerator and gave the 2,4-dinitrophenylhydrazone monohydrochloride, m.p. 213–214° (dec.), yield, 1.83 g.

The N-acethylamino ketone (II) (12.59 g.) was dissolved in 30% sodium hydroxide solution and steam-distilled into a flask containing a small volume of hydrochloric acid. The distillate was concentrated under diminished pressure to yield light yellow needles. The needles were recrystallized from a mixture of methanol-ethanol (1 : 6 V/V) and gave colorless needles melting at 196–198°. Yield, 14.04 g. The melting point was not depressed upon admixture with pseudoöxynicotine dihydrochloride prepared by Pinner and Wolfenstein's method[4)].

Anal. Calcd. for $C_{10}H_{14}N_2O \cdot 2HCl$: C, 47.82; H, 6.42; N, 11.15. Found: C, 47.57; H, 6.40; N, 11.22.

(II) was also obtained by refluxing the dihydrochloride of (III), prepared by Pinner and Wolfenstein's method[4)], with acetic anhydride and anhydrous sodium acetate. This compound (II), boiled at 208–210°/4.5 mm.

Anal. Calcd. for $C_{12}H_{16}N_2O_2$: C, 65.43; H, 7.32; N, 12.72. Found: C, 64.98; H, 7.53; N, 12.78.

The 2,4-dinitrophenylhydrazone of this compound (II) was prepared in the same way as described above and melted at 213–214° (dec.). The mixed melting point with the hydrazone obtained above showed no depression.

Takuro Kisaki
Masanao Ihida
Einosuke Wada

Central Research Institute, Japan Monopoly Corporation, 2-1390, Yutaka-machi, Shinagawa-ku, Tokyo, Japan

Received June 18, 1959

ERRATA

page	*column*	*line*	*for*	*read*
Vol. 23, No. 4				
284	left	7	solotion	solution
287	left	12	raction	fraction
287	right	22	not were	were not
294		FIG. 8	Aminomethylfurfural	Aminomethylfurfural Hydrochloride
301	right	7	XV	XIII
301	right	8	XVI	XIV
301	left	under structural formula		XIII
301	right	under structural formula		XIV

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 33, No. 7 (1959)

Studies on the Classification of Baker's Yeast. Part IV. Comparison of Growth Curve of Each Strain.

(p. *505~507*)

By Tatsuro YAMAGUCHI
(*Institute of Applied Microbiology, University of Tokyo*)

Growth curves of modified Olson-Johnson's medium were carefully compared, using 30°C Mond's shaking apparatus and L-form tube under 0.60 m mol O_2/l,min. effective aeration, in order to know if there are any discrepancies in growth phase among morphologically and physiologically different baker's yeast strains. Amount of growth were measured nephelometrically and growth curves were expressed by terms of hourly modulus H, lag time and max. growth.

There were substantially no difference in growth curve among commercial strains and, under above condition, majority of them showed 1.68~1.75 of H, that is growth coefficient r=0.52~0.56, 1.0~2.0 hours of lag time and around 12.0 g/l of max. growth in 24 hours culture. Some of the collection strains showed remarkably lower H, longer lag time and lower max. growth than above.

Studies on the Classification of Baker's Yeast. Part V. Vitamine Requirement Tests. (p. *508~513*)

By Tatsuro YAMAGUCHI (*Institute of Applied Microbiology, University of Tokyo*)

Vitamine requirements of baker's yeasts in modified Schultz and Atkin's solutions, using Difco's vitamine-free casamino acid as N source, were tested. Cultivation was done by 30°C Monod's shaking culture apparatus using L-form tube and inoculum was 10^3 cell/cc in each test medium.

These baker's yeasts showed five growth curve patterns in various incomplete media, and among them those of commercial baker's yeasts were two. Majority of commercial yeast required Ca-pantothenate and Biotin as vitamine, but 5 of commercial baker's yeasts required B_1 or B_6 in addition. These difference in growth curve pattern were also observed in stationary culture. Any efforts to find more detailed discrepancies in vitamine requirement were failed, although some of them showed lower growth in inositol-less medium.

Thus difference in vitamine requirements was found to be a very useful key for classifying the baker's yeast strains. Classification of baker's yeast based upon the characteristics stated both in previous and this papers will be reported in next paper.

Studies on the Classification of Baker's Yeast. Part VI. Classification of Baker's Yeast of the World.

(p. *513~519*)

By Tatsuro YAMAGUCHI
(*Institute of Applied Microbiology, University of Tokyo*)

Based upon the results of various experiments studied in previous papers, classification were made among 79 baker's yeast strains collected from 12 countries. Using vitamine requirements and macromorphological characters as principal keys, these baker's yeast were classified into groups, and all commercial baker's yeast were found to belong into 23S, 23I, 236I, and 236R groups. Each of these groups was also reclassified according to the other morphological and physiological keys and characteristics of each group and the belonging strains were precisely described.

Majority of commercial yeast, all of the Japanese and some of the other 10 countries' yeast, belonged to 23S group. However, 8 yeast from certain samples of U.S.A., W. Germany and Denmark belonged to 23I group, and one yeast from Holland to 236I group. Also four strains from American Baker's yeast company belonged to 236R group. Majority of 23S strains were high sugar soln.—1) and high sugar dough—2) fermenter and seemed to be baker's yeast race suitable for high sugar containing bread, and strains belonging to 236I and 236R groups were non-high sugar soln.—and low sugar dough—fermenter and seemed to be baker's yeast race suitable for ordinary bread.

Discussion was made upon various subjects of yeast strain and also upon the general characteristics of baker's yeast.

1) T. Tanemura, Personal communication.
2) T. Sato et al., Personal communication.

Chemical Studies on Toxic Protein "Ricin" in Castor Bean. Part III. The Chromatographic Fractionation of Ricin Tb. (p. *520~524*)

By Gunki FUNATSU (*Biochemical Laboratory, Faculty of Agriculture, Kyushu University*)

Ricin Tb was fractionated into three components—Tb-I, Tb-II and Tb-III—by calcium phosphate column chromatography with phosphate buffer of pH 6.0. This Tb-I came out at the concentration of 0.15M, Tb-II at 0.20M and Tb-III at 0.30M phosphate buffers respectively during the chromatography. All these three components were homogenous ultracentrifugally and electrophoretically. It was shown that Tb-I was a high toxic protein which had no proteolytic activity, Tb-II was a high toxic protein which might have proteolytic activity and Tb-III was a non-toxic protein which had high hemagglutinating property.

Studies on the Enzymes of Lactic Acid Bacteria. Part XII. Characterization of Racemiase Reaction.

(p. *524~528*)

By Kakuo KITAHARA and Akira ŌBAYASHI
(*Institute of Applied Microbiology, University of Tokyo*)

The extraordinarily small turnover number of coenzyme DPN as well as very loose affinity between enzyme and coenzyme are, as already pointed out, evidences which show the curiousness of racemiase reaction.

In this paper several other physiochemical constants were searched in order to obtain some informations upon the mechanism of lactate-racemization.

Purified specimens of apo- and co-racemiase, both derived from *Lactobacillus plantarum*, were acted upon D- and L-lactate and obtained following results.

Dissociation constant for enzyme-substrate complex, Michaeli's constant: $Km=2.8\times10^{-2}M$; optimum pH: 7.0~7.5; optimum temperature: 35°C; energies of activation for L-lactic acid and D-lactic acid were almost equal, 6,500 and 7,100 calories respectively; equilibrium constant: $K=\frac{[\text{L-lactate}]}{[\text{D-lactate}]}=1$.

Pyruvate, mandelate, adenosine triphosphate, adenine diphosphate, nicotinic acid and its amide inhibit the racemiase reaction. The effect of the first two may be caused by their competition against substrate lactate, while the effect of others by their competition against coenzyme DPN.

Studies on the Enzymes of Lactic Acid Bacteria. Part XIII. Production of *l*-Malic Acid by the Fumarase of *Lactobacillus brevis*.

(p. *528~531*)

By Kakuo KITAHARA, Sakuzo FUKUI and Masanaru MISAWA
(*Institute of Applied Microbiology, University of Tokyo*)

With the intension for producing *l*-malate enzymatically from fumarate, we recognized again that *Lactobacillus brevis* is most suitable for this purpose among lactic acid bacteria.

The equilibrium constant *K* of fumarate, [*l*-malate]/[fumarate], was found to vary according to the experimental conditions. For example, in the case of Ca-salts, the value of *K* at 30°C and pH 5.8 was determined as 10.3 instead of 3.3 which had been known with Na-salts.

This finding is convenient for producing malic acid, however, the solubility of Ca-fumarate, 7.8×10^{-3} mol., is too small for practical application. But it is important to note that the solubility of Ca-*l*-malate is also quite small, 5.2×10^{-3} mol.

If fumarate were added in the water suspension of Ca-fumarate, the salt in solid phase would dissolve successively into the liquid phase as the enzymic reaction proceeded, and *l*-malate formed here would be obliged to crystal out, because its solubility is too small for retaining the equilibrium with fumarate. Thus, in spite of its reversibility, the fumarase reaction would continue one-sidedly until the last crystallet of Ca-fumarate vanished away.

Calcium fumarate corresponding to 50 g. of free acid and 0.5 g. of dried cells of *L. brevis* were suspended in 1 liter of distilled water and agitated gently for 24 hours at 30°C. At the end of this reaction time, the precipitate obtained by filtration was known to be almost pure crystals of Ca-*l*-malate amounting 74.5 g. (50.5 g. free acid).

Studies on Sugar-Dehydrogenase of Mould. Part VI. Purification and Some Properties of Glucose Dehydrogenase.

(p. *532~536*)

By Humio KURASAWA and Ikuo IGAUE
(*Faculty of Agriculture, Niigata University*)

Glucose dehydrogenase from *Aspergillus oryzae* (Korean-B) has been purified and the separated enzyme component was estimated to be electrophoretically homogeneous.

The extraction from triturated mycelium of *Aspergillus oryzae* with 0.1M phosphate buffer (pH 8.2) was followed by fractionation with ammonium sulfate between 60 and 100 per cent saturation. The precipitate was dissolved in water and dialyzed for two days.

To the solution, ortho aluminium hydroxide α gel was added to absorb the enzyme, and the gel was eluted with 0.2M phosphate buffer (pH 7.8). The second absorption followed.

The total elutant was fractionated with ammonium sulfate between 75 and 100 per cent saturation.

Further purification of the enzyme was achieved by electrophoresis using a preparing cell.

The resulting solution had an activity about 40 times greater than that of the original extract.

The yield was 9.1 per cent; Q_{O_2} (protein) 360; electrophoretic mobility 2.4×10^{-5} cm^2. volt^{-1}. sec^{-1} at pH 6.8; isoelectric pH 4.6 and protein nitrogen 16.85 per cent.

Fourteen kinds of amino acids in the hydrolyzate of enzyme were detected by the process of paper chromatography.

Moreover, the flat absorption spectrum of enzyme was observed from 260 mμ to 290 mμ, and there was no particular absorption in the vicinity of 280 mμ.

Studies on Sugar-Dehydrogenase of Mould. Part VII. Chemical Properties of Glucose-Dehydrogenase.

(p. *536～540*)

By Humio KURASAWA and Ikuo IGAUE

(*Faculty of Agriculture, Niigata University*)

Glucose dehydrogenase extracted and purificated from mycelium of *Aspergillus oryzae* (Korean-B) has been investigated in the present paper.

Optimum action of the enzyme was at pH 7.8, 30°, and aqueous solution was rapidly inactivated by heat treatment at 55°, 5 minutes.

Michaelis constant (Km) of the enzyme was 1.1×10^{-2}M at pH 7.8 by Lineweaver-Burks' method.

On the redox-indicators, 2,6-dichlorphenol indophenol could be rapidly reduced by the enzyme about four times as fast as thionine, while methylen blue was not reduced at all.

Enzyme action was remarkably inhibited by heavy metals, slightly by ethylurethane, but not inhibited by NaF, CH_2ICOOH, Na_3AsO_3 and NaCN. Further, its action was almost uneffected by hydroxylamine and *o*-phenylenediamine.

Glucose as substrate was oxidized at a greater rate, xylose, galactose and mannose followed. Sugar acids failed to stimulate the decolorization of the redox-indicator.

Thus, glucose dehydrogenase was separated, but the active group of the enzyme was not investigated sufficiently.

Then, at various points, this enzyme was different from Müllers' glucose oxydase and Frankes' aeroglucose dehydrogenase, and another glucose dehydrogenase may exist in the *Aspergillus oryzae*.

Biochemical Study of Rice Starch. Part VII. The Property of Enzyme Q-Fraction which converts the Blue Color of Amylose-Iodine Reaction to Red at the Milky Stage of Rice Seed Maturation.

(p. *541～546*)

By Humio KURASAWA, Ikuo IGAUE and Tosiro HAYAKAWA

(*Department of Agriculture, Niigata University*)

Enzyme Q-fraction was prepared from seedrice at the milky stage of its maturation by precipitation with lead acetate followed by salting out with ammonium sulfate.

When amylose is attacked by the enzyme, the blue color of the iodine reaction is converted to red without decoloration during subsequent 24 hours, and the reducing sugar formed is 14 per cent. Optimum pH of the enzyme action is 6.7 and optimum temperature 33～34°. Analysis by the electrophoretic method of Tiselius shows that at least three components are present. The enzyme treated with heat does not show any activity towards amylase.

Biochemical Study of Rice Starch. Part VIII. The Products from Amylose by the Action of Enzyme Q-Fraction.

(p. *546～550*)

By Humio KURASAWA, Ikuo IGAUE and Toshiro HAYAKAWA

(*Department of Agriculture, Niigata University*)

Enzyme Q-fraction was prepared from seedrice. By the action of enzyme Q-fraction the blue color of amylose-iodine reaction was converted to red without decoloration followed. The properties of a products prepared from amylose by action of enzyme Q-fraction were studied. The products are non-reducing, does not retrogradate from solution, and stains red in color with iodine. The products are assumed to be amylopectin by the experiments on iodine adsorption, potentiometric titration with iodine and degree of degradation by β-amylase.

Penicillin Production by Continuous Feeding.

(p. *550～554*)

By Shozo UNO, Eitaro KOMATSU, Yoshio OKAYASU, Yasuhiro SAKAKI, Nobuyoshi GOTO and Daikichiro NISHIKAWA

(*Central Research Laboratory, Mitsubishi Chemical Industries, Ltd.*)

Using jar-fermentors of the type similar to those used by Drs. Hosler and Johnson, fundamental research has been made on penicillin production by continuous feeding of carbon and nitrogen sources in the presence of corn step liquor. As a starting medium, very simple medium containing 6% corn step liquor, 1% glucose and a small amount of mineral matters were used.

The experimental results showed:

(1) The optimum pH value of fermenting broth lies

between 6.6 and 6.9.

(2) When glucose is used as carbon source, the feeding rate should be 0.06% per hour.

(3) For a source of nitrogen the feeding of 0.005% of ammonium sulfate per hour will do.

(4) Using an inoculum in a tank, a straight potency increase assending to 5,000 u/ml in 120 hours and to 6,000 u/ml in 144 hours can be obtained.

In this case, the feeding rate of glucose is increased by 50%, to 0.09 per hour, after 72 hours' fermentation.

Studies on the Taka-α-amylase. Part VII. Properties of Crystalline Taka-α-amylase-rivanol Complex. (p. *554~557*)

By Akira TANAKA

(*Laboratory of Food Chemistry, Hyogo University of Agriculture*)

These crystals showed at least two absorption maxima at about 360 mμ and 270 mμ, which almost agreed with those of the mixture of amylase (A) and rivanol (R) (the molar ratio R/A=25).

The rivanol contents of these crystals ranged from 25 to 40 moles per one mole of amylase according to the difference of conditions where these crystals were formed.

Some of rivanol contained were easily removable by washing with water, the R/A ratio then gradually decreased to about 22.

When the washed crystals were resuspended with water, the R/A ratio of both the dissolved and the undissolved part gradually changed respectively; the dissolved decreased and the undissolved increased, while the conc. of rivanol were unchanged. The increased portion of rivanol was easily removable by washing with water. Consequently, this phenomenon may suggest the following equilibria;

$$\begin{tikzcd} \text{R A (cryst.)} \arrow[r, shift left] & \text{R A (sol.)} \arrow[l, shift left] \arrow[d, shift right] \\ \text{R (absorbed)} \arrow[r, shift left] & \text{R+A} \arrow[l, shift left] \arrow[u, shift right] \end{tikzcd}$$

One support of this assumption is brought forward by the fact that the crystal did never show one definite solubility.

The washed crystals contained 1.05 molar equivalent of calcium per 1 mol amylase protein.

Studies on Kojic Acid Metabolism by Micro-organism. Part III. Decomposition of Kojic Acid by Mold *Asp. oryzae* var. *globosus*. (p. *558~563*)

By Tai TOKUYAMA, Seiichi NONOMURA and Chuji TATSUMI

(*Faculty of Agriculture, University of Osoka Prefecture*)

A substrate, kojic acid, was metabolized in both submerged and replacement culture with *Asp. oryzae* var. *globosus*.

In a submerged culture, kojic acid was almost completely decomposed in a few days though any degradation intermediate was not found. Inhibitors such as azide, α,α'-dipyridile and 2,4-dinitrophenol inhibited the degradation of kojic acid.

On the contrary, in a replacement culture, a few organic acids were detected as the metabolic intermediate.

Employing the paper partition chromatography, two substances, ferric ion positive and unstable under light and heat, were also detected but not yet identified.

From the results of the replacement culture, the fungal degradation of kojic acid was suggested.

Studics on the Components of *Ficus carica* L. Part I. Components of the Root. (1) Isolation of Psoralene, Bergaptene and Octacosane. (p. *564~567*)

By Shun-ichi FUKUSHI

(*Department of Agriculture, University of Tottori*)

The alcohol extract of the root of *Ficus carica* L., during the flowering season, yielded psoralene, bergaptene and octacosane.

The isolation of the furocoumarin was effected by the utilization of column chromatography through Al_2O_3.

Studies on the Flavor of Natto (boiled soy beans inoculated with *Bacillus natto*). Part I. Identification of Volatile Acidic Compounds by Paper Chromatography. (p. *567~569*)

By Yataro OBATA and Kagenori MATANO

(*Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo*)

The paper chromatographic techniques were employed to identify the chrracteristic components of Natto flavor.

A light yellow oil (47.59 g.) with a strong pleasant Natto flavor was obtained after extraction of 600 g. of Natto with threefold ethyl ether. The oil was steam-distilled and the distillate was made acidic with sulfuric acid and extracted with ether. The other was evaporated and 0.706 g. of volatile acids was obtained. These acids exhibited characteristic flavors and were identified by paper partition chromatography of hydroxamic acids.

Acetic, propionic, butyric, valeric, caproic and phenyl acetic acids were identified and two unknown spots were noted. These two unknown spots were identified as coming from diacetyl. It is conjectured that diacetyl may be considered as a characteristic component of the

Natto flavors.

Studies on the Flavor of Natto (boiled soy beans inoculated with *Bacillus natto*). Part II. On the Change of Diacetyl Content during the Course of Natto Manufacture. (p. *569～571*)

By Yataro OBATA and Kagenori MATANO

(*Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo*)

Diacetyl produced during the Natto fermentation of boiled soy beans by *Bacillus natto* was held responsible for some of the desirable flavor and aroma in Natto in our previous paper.

When samples were stored 20 hours at 40～43°, it was noted that the diacetyl content of Natto increased gradually to a maximum of 0.15 p.p. m., after which there was a moderate decrease. When these samples stored 16 hours at 40～43° were removed to a maturing room at 18°, the diacetyl content reached a maximum at 32 hours after. Whereafter the diacetyl content decreased gradually and a development of unpleasant odor was recognized.

It was noted that the agreement of the time of the maximum production of diacetyl with the period of the highest desirability for serving as food suggests an intimate connection between the diacetyl content and the taste of Natto.

Studies on the *l*-Malic Acid Fermentation. (p. *571～576*)

By Shigeo ABE, Akira FURUYA, Takeshi SAITO and Kenichiro TAKAYAMA

(*Tokyo Research Laboratory, Kyowa Fermentation Ind. & Co. Ltd.*)

The organic acid accumulation by a number of molds was studied and it was found that accumulation of a large amount of *l*-malic acid had been attained by some strains of *Aspergilli* such as *Asp. parasiticus*, *oryzae* and *flavus*.

The strains used in this study were consist of newly isolated organisms from natural materials and type-cultures offered from several institutes for culture collection. Those fungi were grown aerobically in a medium containing glucose, peptone, salts and $CaCO_3$ on shaking culture devise. *l*-Malic acid produced was extracted and purified. The crystal of the acid has a melting point at 97° to 98°C and other properties of the acid were completely identical with those of an authentic specimen.

The maximum yield of *l*-malic acid in this study was about 30 mg. per ml. of culture broth.

Relation of Iodine Reaction and Polymerlization Degree of Amylose. (p. *576～580*)

By Kazuji OHASHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Gifu*)

Amylose extracted from corn starch with hot water was precipitated by adding butanol to the hot water solution and recrystalized from aquous butanol. Amylose thus obtained combined with 19.4% iodine, when titrated potentiometrically with iodine reagent. Amylose was partially hydrolyzed by dilute hydrochloric acid (pH 2.2) at 100° and the acid was removed by filtration through Amberlite IR 112. Partial hydrolyzates of amylose were fractionally precipitated with potassium iodine and iodine absorbed in free iodine concentration ranging frgm 1.6 to 2.5,3 to 5,5 to 10, and 10 to 20×10^{-6}N, respectively. The lower linear polymers non-precipitated, were fractionally precipitated with alcohol. From periodate oxidation measurements and coloration degree with iodine of linear polymers, amylose of DP of 21 give a red color with a absorption peak at 510 mμ, 28 purple 540, 34 violet 560, 41 blue violet 580, and 61 to 330 blue with 600 to 630 mμ, respectively.

On the Chain Length of Starches. (p. *580～583*)

By Kazuji OHASHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Gifu*)

The amounts of iodine absorbed by the various linear polymers (in previous report) were determined for free iodine concentration of 1.0 to 50×10^{-6}N in solution, when titrated potentiometrically with iodine reagent. The longer linear polymers of DP of 61 to 330 absorb 14.5 to 18.3% iodine in free iodine concentration of 2.5×10^{-6}N. While the middle linear polymers of DP 28 to 41 absorb no iodine in 2.5×10^{-6}N absorb 6～18% iodine in 20×10^{-6}N.

The shorter linear polymers of DP 16 to 22 absorb only 0～0.2% iodine in $20\sim 10^{-6}$N. It is found that potato starch absorbed 4.05% iodine, corn starch 4.90%, and wheat starch 4.80% in 2.5×10^{-6}N, but potato starch absorbed 6.75%, corn starch 5.88%, wheat starch 5.68% in 20×10^{6}N. These experimental evidences from amount of the iodine absorbed by linear polymer and natural starches suggest that the content of amylose of potato starch is smaller than that of corn and wheat starches, but middle linear polymer of potato starch is more than that of corn and wheat starches.

Behavior of Amylodextrin with Amylose Complexing Agents. (p. *583~586*)

By Kazuji OHASHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Gifu*)

The reagents chosen for the investigation were *n*-butyl alcohol and isoamyl alcohol. When amylose is acid hydrolyzed to a DP of about 40~50, amylodextrin of DP of about 34 can no longer form insoluble complexes with butanol. It is still capable of forming an insoluble complex with isoamyl alcohol, but about 28 can no longer be precipitated by addition of isoamyl alcohol.

Determination of Phosphorylase Activities in Plants. Factors Affecting Phosphorylase Proteins.

(p. *586~591*)

By Michinori NAKAMURA

(*Department of Agricultural Chemistry, the University of Tokyo*)

In the determination of phosphorylase activities in plants, many enzymes and substances which are present in plants interfere more or less seriously with the determination. Among these interfering factors, those affecting the phosphorylase proteins are the main concern of this paper. An example is given which shows that phosphorylase activities are dependent on the cultivation conditions, the time at which samples are collected, and the tissues tested. Phosphorylase in plants is easily extracted with water, and most of its activity is present in the supernatant fraction after the crude extract is centrifuged at 12,000×g. for 10 min. The stability of phosphorylase in crude extracts differs very much from species to species; phosphorylase activities in crude extracts from leaves are easily inactivated even these extracts are stored in the cold. Added adenylic acid has no stimulating effect on such inactivated extracts. Also, there is no indication for the presence of an PR-enzyme. Protease activities are not detected in crude extracts from some plants. Added papain has no inactivating effect on phosphorylase activities of these extracts, except that from spinach leaves. Effects of natural inhibitors present in plants are discussed.

Volume Fraction of Cell Suspensions.

(p. *592~596*)

By Syozo KOGA, Chie KURIBAYASHI and Kazuko NUNOMURA

(*Institute of Applied Microbiology, University of Tokyo*)

A rheological method for the evaluation of volume fraction (ϕ) of cell suspensions was discussed with special regard to *Saccharomyces cerevisiae*. Experimental results are able to be fitted by a quadratic expression of ϕ, as expected by Einstein's formula for a simple, rigid and spherical suspension. The coefficients seem, however, to assume values different from the theoretical ones. As examples of direct application, volume growth curves and osmotic volume changes were shown giving, thereby, 1.25×10^{-1} mol/l as the equivalent osmotic pressure of a yeast cell. The correct value of ϕ thus obtained might also serve to compute various other cell quantities from the observed physico-chemical properties of a whole suspension.

Studies on the Anomalous Linkage in Starch. Part V. "z-Enzyme in Emulsin" is decided to be an α-Amylase. (p. *596~602*)

By Akira BABA (*Shiga Agricultural College*)

The nature of "Z-enzyme" and its action on amylose were investigated employing the partially purified apricot emulsin which prepared by fractional precipitation with tannin, ammonium sulfate, alcohol and acetone.

Amylose, the substrate, is degraded by the enzyme, and fragments of comparatively low molecular weight are yielded which retrograde more rapidly than original amylose.

The enzyme, furthermore, produces glucose, maltose and oligosaccharides from amylose, and the degree of saccharification (Rm-value) determined at the achroic point of the iodine staining of a reaction mixture indicates about 30%.

On the other hand, buffered (pH 5.0) emulsin solutions containing or not containing 0.012M calcium acetate are heated at 60°C. In this treatment, amylolytic action of emulsion solution not containing calcium acetate is destroyed completely within 10 minutes, while the amylolytic action of the solution containing calcium acetate remains about half even after 30 minutes.

Applying this calcium treatment, "Z-enzyme" not containing β-glucosidase and β-xylosidase is prepared.

From the experimental result above mentioned, "Z-enzyme" in emulsin is concluded to be an α-amylase.

Synthesis of β-Alkyl-cellobiosides (acetate).

(p. *602~604*)

By Yoshiyuki INOUE and Ikuo KARASAWA

(*Department of Biochemistry, College of Agriculture, Kyoto University*)

The syntheses of β-cellobioside heptaacetates of some alcohols, in order to use them as the starting materials in the study of transformation of β-cellobiosides into α-

form, have been studied.

Out of the procedures investigated, it seems to be convenient for the preparation of β-celloboisides to react acetobromocellobiose with alcohols, in chloroform or benzene solution, in the presence of silver carbonate.

The β-cellobiosides obtained in the present study are as follows: β-ethyl-cellobioside heptaacetate, m.p. 185.5°~186.5° (uncorr.), $[\alpha]_D^{11}$-23.3° (chlfm.), β-isopropyl cellobioside heptaacetate, m.p. 198° (uncorr.), $[\alpha]_D^{12}$-22.4° (chlfm.), and β-*n*-butyl cellobioside heptaacetate, m.p. 184~185° (uncorr.), $[\alpha]_D^{12}$-24.1° (chlfm.).

Studies on the α,β-Transformation of Glycosides. Part X. Transformation of β-Alkyl-cellobioside Heptaacetates into α-Form. (The Syntheses of α-Alkyl-cellobioside Heptaacetates.) (p. *604~606*)

By Yoshiyuki INOUE and Ikuo KARASAWA

(*Department of Biochemistry, College of Agriculture, Kyoto University*)

The α-isomers were prepared through the transformation of β-isomers with the aid of antimony pentachloride or titanium tetrachloride, and it was found that selenium tetrachloride was, on the contrary not effective.

β-Alkyl-cellobioside heptaacetates were treated with titanium tetrachloride or antimony pentachloride in chloroform solution in various conditions.

The β-cellobiosides obtained in the present study are as follows: α-ethyl-cellobioside heptaacetate, m.p. 175° (uncorr.), $[\alpha]_D^{15}+57.14°$ (chlfm.), α-isopropyl-cellobioside heptaacetate, m.p. 211° (uncorr.), $[\alpha]_D^{13}+59.82°$ (chlfm.), α-*n*-butyl-cellobioside heptaacetate, m.p. 172° (uncorr.), $[\alpha]_D^{13}+55.31°$ (chlfm.).

The Effects of Intake Levels of Dietary Protein on the Animals. Part I. Changes in Some Organs and Nitrogen Constituents of Urine of Rats. (p. *606~610*)

By Hiroshi NISHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Utsunomiya*)

Three groups of young rats were fed on the diets of 10, 20, 30% protein levels for 3 to 5 months, and growth curve, weights of some organs and urine constituents were compared. Body weights of the adult rats fed on 30% protein diet were the least of all. Weight of kidney and hepatic nitrogen were higher amounts of dietary protein.

Every nitrogen constituent of urine was plotted against total nitrogen of urine. Increase of urea nitrogen was proportional to total nitrogen in the whole region. The curve of allantoin nitrogen did not rise linearly, but approached slowly to a certain constant level. A certain correllation between creatinine nitrogen and total nitrogen was possible to be recognised. Uric acid nitrogen were nearly on the constant level on the whole region.

The most rapid growth was shown by rats fed on 20% protein. The total nitrogen of urine of this group was on the region where allantoin nitrogen approached to a constant level. This matter shows that excreted allantoin nitrogen perhaps may be one indicator of optimum level of protein.

Proteolytic Enzymes of Butyl Bacteria. Part IV. The Effects of Metal Ions on the Proteinase of Acetone-Butanol Bacteria. (p. *610~615*)

By Shinji DOI, Yasuyuki KANEKO, and Fuji UCHINO

(*Department of Agricultural Chemistry, Faculty of Agriculture, Nagoya University*)

The effects of some metallic ions upon the proteinase of the acetonebutanol bacteria, strain No. 314, were investigated and following results were obtained.

1. Ethylenediaminetetraacetate (EDTA) completely inhibits the proteolytic activity against milk casein at the level of 10^{-4}M, and this recovered reversibly by the addition of Co^{++} (10^{-4}M).

2. On the incubation of enzyme with EDTA at relatively high temperature, the degree of reactivation of enzyme caused by Co^{++} is low, on the other hand, at low temperature, the reversion is almost quantitative.

3. The inactivation by dialysis of enzyme against EDTA is recovered with some metals (Co^{++}, Mn^{++}, Zn^{++} etc.), especially Co^{++} and Mn^{++} are very effective.

4r Mn has protective effect on the inactivation of intact enzyme, and Co^{++}, Mn^{++}, Z^{++} and Ca^{++} for EDTA-treated enzyme.

5. This experiment suggests that the inactivation by EDTA-treatment is reversible and that of EDTA-inhibited enzyme is irreversible.

$$\text{Native Enzyme} \underset{\text{Metal}}{\overset{\text{EDTA}}{\rightleftarrows}} \text{Inactive Enzyme}$$

$$\longrightarrow \text{Denatured Enzyme}$$

X-Ray Diffractmetric Studies on Starches. (4) Formation of Native Starch-like Granules of Amylodextrin in Vitro. (p. *615~619*)

By Susumu HIZUKURI, Ziro NIKUNI, Yasutsuna HATTORI* and Shusaku WADA*

(*The Institute of Scientific and Industrial Research, Osaka University; *Matsutani Kagaku Kogyo Kaisha Ltd.*)

A crude linear amylodextrin (Amylodextrin I) was separated as white precipitate from a commercial sweet potato syrup manufactured for new process of Saké-brewing. Amylodextrin I was regular small masses without birefringence, and gave very sharp A-type X-ray diffraction pattern. The average degree of polymerization of Amylodextrin I was 12.0 and it's $[\alpha]_D^{20}$ was +187.5°.

By recrystallization of Amylodextrin I from an alcoholic solution (methanol: *n*-propanol: water=3:3:4, by volume), regular small granules (Amylodextrin II) with birefringence polarization crosses were obtained. These granules gave A-type X-ray diffraction pattern. The average degree of polymerization of Amylodextrin II was 15.0, it's $[\alpha]_D^{20}$ was +196° and λ_{max} of it's iodine coloration was 485 mμ. On the other hand, by recrystallization of Amylodextrin I from an aqueous solution (ca. 33%), simillar granules as Amylodextrin were obtained. However, these granules (Amylodextrin III) gave B-type X-ray diffraction pattern. Other physical properties were shown almost same with Amylodextrin II.

Amylodextrin II and III, thus obtained, were considered to have the same fine structure as native cereal and tuber starch granules, respectively. They had, however, more higher crystallinity than the native starch granules as shown by the comparison of X-ray diffractometer curves.

The difference between crystallinity and retrogradation tendency of amylose component was discussed.

Nutritional Studies on Fluorine Part I. Modified Micromethod of Fluorine Determination by Using of Aluminium-Hematoxylin Lake. (p. *619~623*)

By Susumu FUJIE

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

For fluorine determination, several methods have been reported. Among them, aluminium-hematoxlin method which is based on the very sensitive reaction between aluminium-hematoxylin lake and fluorine ion, seems to be suitable for microdetermination of fluorine. In this method, unfortunately, fluorine is determined by observing different colors developed in accordance with fluorine content by the naked eyes. The improvement desired was of this point.

The method developed in this study is as follows: 6.5 ml. of 0.005% aluminium aqueous solution is diluted to 500 ml. with distilled water, then 10 ml. of saturated ammonium carbonate solution and 6.5 ml. of 0.1% hematoxylin acetate solution are added to that solution. After 20 minutes at 30°, 20 ml of the mixed solution is added to 50 ml. of acidified fluoride solution (containing below 10 γ of fluorine) by 2 ml. of 1N acetic acid. Afler 20 minutes exactly, the absorbance is determined at a wave length of 550 mμ on a Beckman spectrophotometer. This method allows the reproducible estimation of fluoride in concentrations as low as 0.01 p.p.m.

Fluorine content in bones, teeth, hairs and milk of cattles, plants and drinking water of some areas have been determined by this method.

Studies on DL-Forming Lactic Acid Bacteria. Part I. Requirements for Organic Bases and Nicotinic Acid, and Formation of Racemiase.* (p. *623~626*)

By Akira ÔBAYASHI and Kakuo KITAHARA

(*Institute of Applied Microbiology, University of Tokyo*)

All the DL-forming lactic acid bacteria used in this research, i.e., *Lacobacillus plantarum* (2 strains), *L. arabinosus*, *L. japonicus*, *L. brevis*, and *Pediococcus hennebergi* did not require purine and pyrimidine bases, but needed nicotinic acid as an essential growth factor. Their fermenting capacity increased in pallalel with the nicotinic acid concentrations within a range of 0~0.1 γ/ml.

The formation of diphosphopyridine nucleotide (DPN), which is the coenzyme of racemiase, appears to be directly dependent upon the concentration of nicotinic acid. When the DL-formers were cultivated in a medium containing a restricted amount of nicotinic acid, they produced the mixtures of DL- and optical active (D- or L-) lactic acids. This can be attributed to the insufficient formation of the coracemiase, DPN, as racemiase activity was restored when DPN was supplied to the nicotinic acid-restricted cells.

The characteristic phenomena thus revealed by the inhibition of DPN formation offer an important clue in classifying the DL-forming lactic acid bacteria: *L. plantarum*, *L. arabinosus*, *L. japonicus* and *L. brevis* are intrincically D-formers, while *L. saké* and *Pediococci* are L-formers.

---

* Studies on the Enzymes of Lactic-Acid Bacteria. Part XIV.

On the Production of Hydrogen Sulphide by Sake Yeast. Part II. The Production of Hydrogen Sulphide by the Suspension of the Pantothenate-deficient Cells. (p. *626~631*)

By Ryôji KODAIRA and Teijirô UEMURA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

The cells of Sake yeast strain, Kyôkai No. 6 harvested at the early stage of its logarithmic growth phase when cultured in the pantothenate-deficient medium, could produce H_2S during their incubation at 30° for 3 hrs in the ammonium sulphate-glucose medium lacking pantothenate. This H_2S production was prevented by the addition of either of L-methionine, β-alanine, and pantothenate, while in contrast enhanced by the addition of either of L-glutamic acid, L-aspartic acid, and L-alanine. L-Leucine, which was previously recognized to have a reversal effect upon the H_2S production in the culture of this yeast in the pantothenate-lacking ammonium sulphate medium, showed no appreciable prevention in this case.

The cells obtained from the pantothenate-deficient culture could produce H_2S vigorously from L-cysteine which was added instead of ammonium sulphate at the above incubation, and L-methionine when added together with L-cysteine, reversed, though not thoroughly, this H_2S production.

A possible scheme of the metabolically controlling role of L-methionine in the sulphur metabolism of this yeast was assumably mentioned.

On the Fat-globule Membrane Materials of Cow's Milk. Part III. The Properties of Lipoproteins in Cow's Milk and their Relations to Phosphatase and Xanthine Oxidase. (p. *631~635*)

By Rinjiro SASAKI and Susumu KOYAMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

It is generally recognized that that the fat-globule membrane consists chiefly of a lipoprotein complex. It was also shown by recent studies that alkaline phosphatase is associated with lipoprotein particles in milk.

At the present, however, many questions concerning the lipoproteins of milk remain unsolved.

In the work described in this paper lipoproteins were isolated from cream using such techniques as salting-out and ultracentrifugation, and their electrophoretic and enzymatic properties were studied.

It was found that milk contained two or three lipoproteins. From results obtained by the paper electrophoretic technique, one of these lipoproteins showed a similar mobility to that of one component of casein, while the other two lipoproteins hardly migrated on paper electrophoresis.

Each of two or three lipoproteins had both alkaline phosphatase and xanthine oxidase activities.

On the Fat-globule Membrane Materials of Cow's Milk. Part IV. The Fat-globule Membrane Lipoprotein and its Enzymatic Properties. (p. *635~638*)

By Susumu KOYAMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Previous paper(1) showed that milk contained two or three lipoproteins, and these lipoprotein fractions had both alkaline phosphatase and xanthine oxidaie activities.

In this paper the fat globule membrane lipoprotein was isolated by ultracentrifugation of mixture of butter milk and butter serum separated from the washed cream.

As expected from the results reported previously, the fat globule membrane lipoprotein had the alkaline phosphatase activity. But the greater part of original enzyme activity of non-wasned cream was removed into washed water during the washing process. The enzyme activity of four times washed cream was no more than 13% of that of non-washed cream.

Of the xanthine oxidase, contrary to expectation, authors could not detect the enzyme activity in the washed cream.

The fat globule membrane lipoprotein hardly migrated on the paper-electrophoresis.

After treatment of the fat globule membrane lipoprotein with alcohol and ether, about 80% of the alkaline phosphatase activity remained in lipid-free proteineous residue. The alkaline phosphatase activity of this residue was also activated by lactose as shown in the previous paper.

(1) R. Sasaki and S. Koyama, *J. Agr. Chem. Soc. Japan*, **33**, 631 (1959).

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 33, No. 8 (1959)

Unfermentable Sugars in the Mash of Amylo Process using Barley as the Raw Material. Part I.
Detection of a Certain Oligosaccharides. (p. *639~643*)

By Hideo ONO and Michio DAZAI
(*Fermentation Research Institute, Agency of Industrial & Technology*)

The residual sugar in the barley mash fermented by the amylo process was always rather much remained compared with others. And by paper-chromatography of it, three major un-known constituents (sugar a, b and c), except pentosyl oligosaccharides, was recognized clearly. So each of these three un-known sugars were fractionated from the carbon column chromatography.

Acidic hydrolysis of sugar a, b and c gave only glucose on the paper-chromatogram, and their polymerization degree calculated from the increase of reducing power was 2.0, 2.8 and 4.1 respectively. By partial acidic hydrolysis of sugar b and c, the former gave sugar a and cellobiose, the latter gave a, cellobiose and b, in addition to glucose and parent sugar, respectively. Emulsin converted sugar a to glucose, also b to glucose and a, (cellobiose was not detected), but did not act on c. And α- and β-amylase attacked none of these three sugars. Further more, sugar a presented the same R_F value with laminaribiose.

By mentioned above, it was thought that sugar a was to be laminari-biose, sugar b and c was tri- and tetra-saccharide containing at least each of one β-1,3-and one β-1,4-linkage. These glucosyl oligosaccharides were seemed to be derived from raw-materials.

Unfermentable Sugars in the Mash of the Amylo Process using Barley as the Raw Material. Part II.
Isolation of Laminaribiose. (p. *644~645*)

By Hideo ONO and Michio DAZAI
(*Fermentation Research Institute, Agency of Industrial & Technology*)

Sugar a, mentioned above, was crystallized from methanol. The crystals were recrystallized from the same solvent: m.p. 205~208°C, un-changed on admixture with an authentic specimen of α-laminaribiose, $[\alpha]_D^{16} +24.8° \rightarrow +18.8°$ (*c*, 2.5 in water).

Acetylation of a portion of the free sugar yielded the crystals. The crystalline acetate was recrystallized from 95 per cent ethanol: m.p. 160.5~161.0°C, undepressed on admixture with an authentic β-octaacetate, $[\alpha]_D^{16}$ $-28.8°$ (*c*, 2.5 in chloroform).

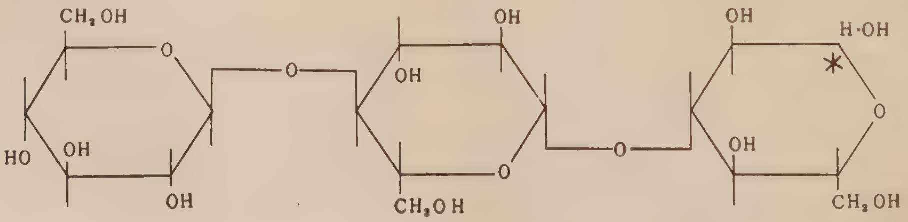
And also these physical constants agreed with that of the pure α-laminaribiose and β-leminaribiose-octaacetate described on the literature, so sugar a was confirmed to be laminaribiose.

Unfermentable Sugars in the Mash of Amylo Process using Barley as the Raw Material. Part III.
Isolation and Structure of a New Gluco-triose. (p. *645~648*)

(*Fermentation Research Institute, Agency of Industrial & Technology*)

Sugar b was presumed to be tri-saccharide from its R_F, the elution behavior and the polymerization degree etc. Specific rotation power of sugar b was $[\alpha]_D^{16} +10°$ (equil.: *c*, 3.0 in water). Glucose from acidic hydrolysis of sugar b was identified as β-glcose pentaacetate. Carbon column chromatography of the partial acidic hydrolysate of sugar b produced the mixture of laminaribiose and cellobiose togather with glucose and parent sugar, so this mixture was fractionated by paper chromatography into two components, and both were crystallized by conversion into β-laminaribiose octaacetate and β-cellobiose octaacetate, respectively.

The sequence of linkages was determined by sodium borohydride reduction of sugar b to the sugar alcohol



and partial acidic hydrolysis of the product. Paper chromatographic analysis revealed that the only reducing disaccharide present was cellobiose. This proved that sugar b was O-β-D-glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→3)-D-glucose.

Acetylation was performed with sodium acetate and acetic anhydride at 125～130°C for 40 min. Crystalline hendecaacetate was formed from 95% ethanol: m.p. 182.5～183°C, $[\alpha]_D^{25}$ −21.7° (*c*, 3.0 in chloroform). *Anal.* calc. for $C_{40}H_{54}O_{27}$: C 49.69, H 5.63 per cent, found: C 49.65, H 5.86 per cent.

"Cellamiose" as the name of the above β-linked trisaccharide was proposed to apply by authors.

Studies on the Safrole Derivatives. Part I. Synthesis of Sesamol and 3,4-Methylenedioxy Phenyl Acetone. (p. *648～652*)

By Shiro SUGAWARA

(*Central Research Institute, Japan Monopoly Corporation*)

t-Butyl hypochlorite has been found to be an effective reagent for the preparation of isosafrole chlorohydrin ethers and esters in reactive solvents of alcoholes or acids. Through the steps, 3,4-methylenedioxy propiophenone, sesamol and 3,4-methylenedioxy phenyl acetone has been prepared from them.

Intracellular Distribution of Nitrate Reductase of Cattle Liver. (p. *652～655*)

By Hirohisa OMURA

(*Agricultural Chemical Institute, Department of Agriculture, Kyushu University*)

The cattle liver tissue was fractionated with the isotonic sucrose solution by the differential centrifuge method and the intracellular distribution of the nitrate reductase was surveyed by estimating the activity of the mitochondrial, microsomal and cytoplasmic fractions. It was found that most of the enzymatic activity dissolved in cell sap, although a feeble activity was detected in the particles. Furthermore, some supplemental experiments were also performed.

Studies on Hexokinase. Part I. Preparation of Potato Hexokinase. (p. *655～658*)

By Yoshiyuki INOUE and Tatsuro ITO*

(*Biochemical Laboratory, Department of Agriculture, Kyoto University; *Faculty of Liberal Arts and Education, Shiga University*)

The hexokinase of potato tubers was investigated. It was found that apyrase, the interfering enzyme to hexokinase, was completely inactivated by heating the potato extracts for twenty minutes at 50°, at pH 7.5 under toluene. By this treatment hexokinase preparations which showed higher activities were obtained.

Partially purified hexokinase was obtained by fracttionation of the extracts with ammonium sulfate. It was revealed that the potato hexokinase thus prepared had both glucokinase and fructokinase activities and that the different degree of saturation of ammonium sulfate caused the difference in the relative activities of both kinases. Further purification of the hexokinase preparation gave rise to fructokinase which was completely free of glucokinase activity.

Studies on Hexokinase. Part II. Preparation of Wheat Germ Hexokinase. (p. *659～661*)

By Yoshiyuki INOUE and Tatsuro ITO*

(*Biochemical Laboratory, Department of Agriculture, Kyoto University; *Faculty of Liberal Arts and Education, Shiga University*).

The procedure for preparing hexokinase from wheat germ is presented. It was found that dried wheat germ completely lost its hexokinase activity after storing for fourty days. ATPase was inactivated by about sixty percent by heat treatment (pH 7.5, 50, 20 mins.) and by seventy two percent by the addition of desoxycholate (0.33%). The hexokinase activity was at the highest in a fraction obtained by 0～25% saturation of ammonium sulfate. The hexokinase preparation was found to lose its activity about sixty five percent after storage at −3° for seven days.

Studies on the Chemical Constituents of Tobacco Plant. Part I. Isolation of Chlorogenic Acid from Fresh and Flue-cured Tobacco Leaves. (p. *661～662*)

By Shigeo AKAIKE

(*Hatano Tobacco Experiment Station, Japan Monopoly Corporation*)

Chlorogenic acid (m.p. 205～206°) was obtained by the lead method from ethanol extracts of 10.0 kg of fresh tobacco leaves (Bright Yellow Var.). The yield amounted to 10.0 g or 0.1% of the weight of fresh tobacco leaves. And from 200 g of flue-cured tobacco leaves 0.06 g of chlorogenic acid was isolated.

Studies on the Chemical Constituents of Tobacco Plant. Part II. Determination of Chlorogenic Acid in Leaf Tobacco. (p. *663～666*)

By Shigeo AKAIKE and Sadayoshi YAMADA

(*Hatano Tobacco Experiment Station, Japan Monopoly Corporation*)

A new method for the determination of chlorogenic acid in leaf tobacco was presented.

According to this method, chlorogenic acid was determined by measuring the optical density at $324\,\mathrm{m}\mu$ of the aqueous extract of a sample after removal of caffeic acid by shaking the extract with ethyl ether, and rutin by passing through a column of Amberlite IRC-50 resin (hydrogen form). The percentage of chlorogenic acid content in the sample was calculated, using an $E^{1\%}_{1\mathrm{cm}}$ value of 526 for anhydrous chlorogenic acid.

The content of chlorogenic acid in yellow leaf tobacco measured by this method was always lower than the value obtained by the Slotta and Neisser's iodometric method.

Studies on the Chemical Constituents of Tobacco Plant. Part III. Changes of Chlorogenic Acid Content during Flue-curing of Tobacco Leaves.

(p. *667～670*)

By Shigeo AKAIKE and Sadayoshi YAMADA

(*Hatano Tobacco Experiment Station, Japan Monopoly Corporation*)

The changes of chlorogenic acid content during flue-curing of tobacco leaves (Bright Yellow Var.) were investigated by the ultraviolet absorption method which had been devised by authors.

It was found that the amount of chlorogenic acid did not change during flue-curing. The result obtained in this study coincided with those reported by Frankenburg and by Penn and Weybrew. But it was inconsistent with the reports of Porcsalmy and of Nagasawa who observed an increase of tannin and chlorogenic acid content during curing.

Studies on the Chemical Constituents of Tobacco Plant. Part IV. Isolation of Meso-inositol from Fresh and Flue-cured Tobacco Leaves.

(p. *670～671*)

By Shigeo AKAIKE

(*Hatano Tobacco Experiment Station, Japan Monopoly Corporation*)

5.1 g of meso-inositol occurring in free state was isolated from ethanol extracts of 10 kg of fresh tobacco leaves (Bright Yellow Var.).

Moreover, 0.11 g of meso-inositol was isolated from 300 g of flue-cured tobacco leaves, using the procedure of Philips et al..

Studies on the Constituents of Saké. Part IV. N-Fraction (1).

(p. *671～673*)

By Yoichi OHTAKA

(*The Institute of Physical and Chemical Research*)

Nitrogen distribution among the fractions of Saké separated by ion exchange resins was measured and it was noticed that 40% of total nitrogen of Saké was found in the basic nitrogen fraction adsorbed to IRC-50 (pH 4.7).

Eighty liters of Saké was separated systematically and samples of six fractions were obtained for further investigation.

Studies on the Constituents of Saké. Part V. N-Fraction (2). Isolation of γ-Aminobutylic Acid.

By Yoichi OHTAKA (p. *674～676*)

(*The Institute of Physical and Chemical Research*)

Amino acids in acidic and neutral nitrogenous fractions of Saké were investigated by means of ion exchange chromatograph, and γ-aminobutyric acid was isolated for the first time.

Studies on the Constituents of Saké. Part VI. Amine Fraction; Isolation of Choline and Agmatine.

(p. *676～678*)

By Yoichi OHTAKA and Tsuyoshi OGATA

(*The Institute of Physical and Chemical Research*)

Amine fraction of Saké separated by ion exchange resins was further subdivided into several components by the procedure of preparative paper pack chromatography. Choline, agmatine and arginine could be isolated and identified. Choline was estimated colorimetrically.

Studies on the Constituents of Saké. Part VII. Identification of Acetylagmatine (a New Substance) and Ethanolamine.

(p. *679～681*)

By Yoichi OHTAKA and Katsuhisa UCHIDA

(*The Institute of Physical and Chemical Research*)

In the amine fraction of Saké, separated by ion exchange resins, the unknown substance reported previously, which was isolated by the procedure of paper pack chromatography, was identified with acetylagmatine (a new substance). Moreover, ethanolamine was found in this fraction.

Sulfate-sulfur in Chicken Egg. Part I. Dialyzable Sulfate-sulfur in Fresh Egg.

(p. *681～685*)

By Yoshinobu NONAMI

(*Faculty of Agriculture, University of Niigata*)

Total sulfate-sulfur in yolk, white and yolk membrane was each determined. Sulfate-sulfur in weakly bound form present in yolk and white was also determined by measuring the free sulfate ion liberated on dialysis or dilution of the materials. The results obtained as follows:

Yolk membrane, composed mainly of mucin, contains a considerable amount of sulfate-sulfur and, in yolk, there is no sulfate-sulfur bound to lipids. Sulfate-sulfur in fresh yolk amounts to 19 mg%, but only 1 mg% exists as bound to yolk protein and 8 mg% in a free or very weakly bound form. Sulfate-sulfur in fresh white is 7 mg% and 3 mg% exists in weakly bound form. Variation of sulfate-sulfur content, observed through one year, is greater in white than in yolk.

Sulfate-sulfur in Chicken Egg. Part II. Bound Sulfate-sulfur in Fresh Egg. (p. *685～690*)

By Yoshinobu NONAMI

(*Facutlty of Agriculture, University of Niigata*)

Bound forms of sulfate-sulfur in fresh yolk and white were studied by measuring free sulfate ion liberated during decomposition by acid, alkali and proteases and also during heat denaturation of materials. Free sulfate-sulfur is 4 mg% (based on fresh material) in yolk and 0.6 mg% in white. In yolk, other 4 mg% is absorbed weakly by yolk proteins and 10 mg% absorbed more strongly. In white 2.7 mg% is absorbed and 4.2 mg% may be bound by chemical bond. Bound sulfate-sulfur, both in yolk and white, is liberated more easily by acid. In white, not all bound sulfate-sulfur is linked to proteins directly, sugar or sugars may be interposed between them and it may be also true in yolk.

Sulfate-sulfur in Chicken Egg. Part III. Distribution of Sulfate-sulfur in Proteins of Fresh Egg. (p. *691～696*)

By Yoshinobu NONAMI

(*Faculty of Agriculture, University of Niigata*)

In order to determine which proteins of yolk and white are bound with sulfate radical by chemical bond, proteins of fresh yolk and white and of more than 40 days dialyzed yolk and white were fractionated and dialyzable sulfate-sulfur were determined. The results are as follows;

Livetin in yolk and ovomucoid and ovomucin in white contain chemically bound sulfate. Labile sulfate-sulfur of livetin is liberated during dialysis and its heat-coagulability dtcreases during dialysis. But there is no relationship between these tendencies. Dialyzable fraction of longer time dialyzed white contains nonproteinous bound sulfate-sulfur which is not precipitated by barium chloride.

Sulfate-sulfur in Chicken Egg. Part IV. Distribution of Sulfate-sulfur in Differentiated Parts of Embryonated Egg. (p. *696～700*)

By Yoshinobu NONAMI

(*Faculty of Agriculture, University of Niigata*)

The purpose of this work was to get some informations concerning the fate of sulfur compounds of yolk and white of embryonated eggs during incubation and to make clear the process of decomposition of yolk and white. The freezed contents of incubated fertiled and unfertiled eggs were separated mechanically and 2.5% HCl-soluble sulfate ion and total nitrogen of these parts were determined. Taurin of embryo was also determined. Observations were made on these data and the data concerning total sulfur and sulfurs containing amino acids of these parts which were collected from the previous works. Metabolism of sulfate-sulfur of embryonated eggs is most active in embryo and its surrounding tissues after 330 hours of incubation. Allantoic fluid contains much free sulfate ion and this fluid may act some significant role in the sulfate metabolism of embryonated egg. The experimental evidence indicates that yolk is decomposed already before 120 hours of incubation but a part of white is not decomposed until 400 hours of incubation. Most firmly bound sulfate-sulfur of both yolk and white are freed after 330 hours. In incubated unfertiled eggs, sulfate-sulfur of yolk is freed and transported to white but that of white is not freed until 21 days.

Sulfate-sulfur of Chicken Egg. Part V. Distribution of Sulfate-sulfur in Protein Fractions of Yolk and White in Embryonated Egg. (p. *700～703*)

By Yoshinobu NONAMI

(*Faculty of Agriculture, University of Niigata*)

To demonstrate the processes of decomposition of yolk and white during incubation, vitelline, livetin, ovomucoid and proteins of white other than ovomucoid were fractionated and sulfate of these protein fractions and liberated sulfate ion were determined at 120, 210, 330 and 400 hours of incubation.

Free or weakly bonding sulfur of both yolk and white almost disappeared before 330 hours of incubation. In yolk, sulfate sulfur was formed from other sulfur compounds after 330 hours. Although, until 400 hours, the appearances of a part of yolk and white remained as the same as these of fresh egg, the part of yolk was denatured before 120 hours but the part of white was not decomposed until 330 hours. The main source of sulfate sulfur for sulfate metabolism of embryo after

330 hours was yolk. In embryonated egg, sulfate sulfur was transported from one part to the other mainly in the form of sulfate ion.

Biochemical Studies on the Pleomorphism of Bacteria. Part I. Rôle of Vitamin B_{12} on the Growth and Division of *Lactobacillus delbrückii*. (p. *703~707*)

By Iwao KUSAKA and Kakuo KITAHARA

(*Institute of Applied Microbiology, University of Tokyo*)

A pleomorphic strain, *Lactobacillus delbrueckii* No. 1 in our laboratory, requires vitamin B_{12} as an essential factor both for growth and for cell division.

It has been determined that the concentration of vitamin B_{12} sufficient for growth is 3×10^{-4} μg/ml, while as high as 1 μg/ml is required for normal cell division. This descrepancy is considered to be the cause of abnormal cell elongation of *L. delbrueckii*.

These abnormally elongated cells revealed Gram-negative and their nuclear chromatins were aggregated in large compact granules.

When the abnormal cells were treated with lysozyme in the hypertonic (0.15M) lactose or sucrose solution, all the cells lysed easily from within, without forming protoplasts.

From these results, it is concluded that deficiency of vitamin B_{12} will cause abnormalities upon the nature of the cell surface, especially the centre controlling the cell-division, resulting abnormal cell-elongation.

Studies on the Effects of Some Physical Conditions on the Submerged Mold Culture. Part II. On the Two Types of Pellet Formation in the Shaking Culture. (p. *707~710*)

By Jôji TAKAHASHI and Kôichi YAMADA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The globose mold colonies (so called pellets) formed in the shaking culture were found to be classified into two groups-" flocculent type pellet " and " non-flocculent type pellet ".

The differences between the developmental history of the flocculent type in which the flocculence of germinating spores could be found and that of the non-flocculent type in which the flocculence of spores could not be found, were reported.

Among many culture conditions which were considered to affect on the mycelial growth, in this paper, the effects of inoculum size and medium constituents on the history of both types of pellet formation were investigated respectively.

Furthermore, some of culture conditions which were required to prevent pellet formation and obtain the homogeneous, filamentous suspension of mycelia, were suggested respectively, in both cases of flocculent and non-flocculent type.

Interrelation between Protein and Silicate in Rice Plant. (p. *710~714*)

By Kenichi BABA and Akira BABA

(*Shiga Agricultural College*)

In order to explain the physiological role of silicate in rice plant, intèrrelation between protein and silicate was investigated with the pressed juice obtained from green rice plant:

The pressed juice is centrifuged, and the sedimentary insoluble matter is discarded. Then the resultant soluble proteins are divided into 7 fractions by the stepwise addition of ammonium sulfate (0.1, 0.2, 0.3~0.7 sat.).

The ratio of silicate to protein-nitrogen (Si-N) in each fractions are determined. In this experiment, it is revealed that the fraction precipitated at 0.1 saturation of ammonium sulfate indicates largest Si/N ratio i.e. 0.52.

On the other hand, paperelectrophoresis of the proteins are carried out for examine whether the proteins and silicate migrate separately or not. However, they are moved together. Therfore some complex of protein with silicate seems to be present in rice plant as a soluble form.

Synthesis of Disaccharides by Mercuric Salts. Part II. Synthesis of 2-O-α-D-Glucopyranosyl-D-glucose (Kojibiose). (p. *714~718*)

By Kazuo MATSUDA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

1,2-α-Linked glucobiose (kojibiose) was synthesized by the condensation of 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide and 1,3,4,6,-tetra-O-acetyl-α-D-glucopyranose with mercuric cyanide. The synthesis was carried out in the following three methods: (1) At room temperature in anhydrous nitromethane; (2) In benzene under reflux; (3) By direct fusion without solvent. The synthesized sugar was characterised as its crystalline α- and β-octaacetates (α-form: m.p. 166°, $[\alpha]_D=153$ (chloroform); β-form: m.p. 117°, $[\alpha]_D=113$ (chloroform)). By the method (2), β-sophorose octaacetate was also obtained. By the method (3), β-sophorose octaacetate and an unknown acetate, m.p. 102°, $[\alpha]_D=-17.2$ (chloroform), were obtained besides the main product kojibiose.

Studies on the Disaccharides in Koji Extract and Saké Part V. Isolation and Identification of Kojibiose. (p. *719~723*)

By Kazuo MATSUDA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

By successive carbon and Magnesol : Celite column chromatography, kojibiose was isolated from koji extract and was characterized as its crystalline α- and β-octaacetates. Free sugar was prepared by usual method, $[\alpha]_D=136(H_2O)$. Paper chromatography and paper ionophoresis showed the known characters of 1,2-linked glucobiose. Reducing power of this sugar was 2.67% of glucose (Somogyi method), 40.14% (Willstätter method) and 50.15% (Betrand method). By treatment with phenylhydrazine HCl, a small amount of glucosazone was directly obtained. Infrared spectra of free sugar and acetyl derivatives were also illustrated.

Studies on Wasabi. Part V. On "Powdered Wasabi". (1) Degeneration in Storing. (p. *723~727*)

By Zenji NAGASHIMA, Masaaki UCHIYAMA and Shigemi NISHIOKA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Shizuoka*)

The term "Powdered Wasabi", in Japan, means powdered dry rhizoma of horse radish to which small amounts of mustard, pigment and others are added. When kneaded with water, it gives spicty taste like mustard and "Wasabi", and therefore it is used as a spice. Powdered wasabi and mustard are often degenerated while storing and then even if they are kneaded with water they may show no spicy taste. In this paper the authors describe on the above degeneration. Two causes of degeneration are conceivable; The one is the decrease of mustard oil glucoside, the original substance of the spicy taste, and the other is the inactivation of myrosinase acting on above glucoside. In this experiment, it was clarified that the latter was the principal cause of degeneration, and it was found that inactivated myrosinase was reactivated markedly by adding ascorbic acid. Furthermore the degenerated powdered wasabi was also restored in taste by the ascorbic acid. Moreover, myrosinase was found to be an enzyme having sulfhydryl radical as active centre, and the mechanism of inactivation of myrosinase in storing was deduced. Further experiments were conducted on the effect of temperature, moisture and the stored period upon the degeneration of powdered wasabi while storing.

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 33, No. 9 (1959)

Studies on the Change of the Composition and the Tanning Properties of Skin Tissue. Part XI. On the Influence of Proteolytic Enzymes in Pancreas on the Properties of Unhaired Skin. (p. *729∼732*)

By Yasushi SATO* and Ryo NAKAMURA*

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

With skins from which different amounts of elastin or collagen were removed by digestion, influences of their digestion on the adsorbed tannic acid of skin were experimented. And the following results were obtained.

(1) Without regard to the digestion of collagen, adsorbed tannic acids are decreased according to the increase of digested amounts of elastin. When digested amounts of elastin reach to about 70% or when they reach to about 40% and simultaneously digested amounts of collagen reach to about 5%, about 10% of adsorbed tannic acid are decreased and its decreased amounts was maximum in our experiments. When the adsorbed tannic acid reaches to the minimum, amounts of amino nitrogen found in acid hydrolysate of digested solution become remarkable.

(2) In the digestion process of collagen only, adsorbed tannic acid are increased a little till 0.2% digestion of collagen, but they are re-decreased to initial value on the further digestion. Thinking from the change of combined water, it is considered that the initial change does not depend on the change of the fundamental structure of skin protein, but the latter change depends on it.

* Present address: Dept. of Agr. Chem., Fac. of Agr., Univ. of Nagoya, Anjo, Aichi Prefecture.

Studies on Silk-worm Deoxyribonuclease. Part I. Characterization of the Enzyme by the Effects of pH, Divalent Cations, and Temperature on the Activity. (p. *733∼737*)

By Jun-ichiro MUKAI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

A deoxyribonuclease has been purified about 30 fold from the acetone-dried tissues of silk-worm larvae by repeated 0.5M NaCl extraction and dialysis, butanol treatment, and freeze-drying, and the general properties of the enzyme were studied using Dische's diphenylamine procedure for activity determination. The following results were obtained; the optimal pH and temperature of the enzyme was about 11.5 and 45° respectively, the enzyme was activated by divalent cations such as Mg^{++}, Mn^{++}, and Ca^{++}, and inhibited by citrate^{---} and EDTA, and it was also seen from heating test that the enzyme was rather stable as compared with pancreatic deoxyribonuclease.

Studies on the Reticulo-rumen Digestion. Part XII. Digestion of Rice Xylan in the Rumen of Sheep. (p. *737∼740*)

By Fumio KUMENO and Makoto KANDATSU

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

In order to study the digestion of xylan in the rumen of sheep, 80 g of xylan from rice straw was put directly into the rumen through the permanent rumen fistula. Disappearance of the xylan from the rumen amounted to 70∼80% within 12 hours, and 92∼94% within 24 hours. The introduction of xylan did not produce a rise in volatile fatty acids concentration, but the slower fall than in control may indicate the production of volatile fatty acids by fermentation of xylan. It was shown that molar percentage of propionic acid was slightly higher and that of acetic acid lower than in control, and the ranges of that of acetic, propionic and butyric acid were 69∼73%, 18∼22%, 8∼12% respectively. Introducing xylan into the rumen had no special effect on pH, concentration of ammonia and numbers of protozoa, and quite a small amount of soluble furfural-producing-substances were produced 8 hours after dosage.

Studies on the Reticulo-rumen Digestion. Part XIII. Production of Volatile Fatty Acids from Starch in the Rumen of Sheep. (p. *740∼744*)

By Fumio KUMENO and Makoto KANDATSU

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The following determinations were made on the rumen content during 7 hours after feeding: concentrations of volatile fatty acids, molar percentages of individual volatile fatty acids, concentrations of ammonia, non-volatile fatty acids, total sugars and reducing sugers.

The diets investigated and the daily ration per shcep were as follows. Diet A: a mixture of 200 g starch and 100 g hay. Diet B: a mixture of 200 g starch, and 100 g hay and 120 g soy bean oil meal. Diet C: 100 g hay.

It was shown that the concentration of total fatty acids increased to 9~10 m.mol/(100 ml rumen fluid) 5~7 hours after feeding of diet A or B. This level is higher by 2~3 m.mol than when diet C was given, and ratio of acetic acid to propionic acids decreased gradually as the concentration of total volatile fatty acids increased.

The concentration of butyric acid did not increase on diet A or C, but increased on diet B. The concentration of ammonia decreased markedly on diet A, on the contrary, increased on diet B. Non-volatile acids and reducing sugars were scarcely found throughout the experiments.

Induction of New Genetic Characters by the DNA Treatment in *Streptomyces griceus* Group.

(p. *744~752*)

By Yujiro HARADA

(*Kyowa Fermentation Ind. Co., Ltd.*)

1. *Streptomyces griseus* strain 15 m, (industrial streptomycin-producing strain, resisted to 130,000 units of dehydrostreptomycin sulfate per ml.) and *Streptomyces olivaceus*, strain G, (vitamin B_{12}-producing strain, was sensitive to dehydrostreptomycin sulfate at 5 units per ml) were used in this experiment.

2. A population of *Streptomyces olivaceus*, strain G, exposed to DNA-preparation of *Streptomyces griseus*, strain 15 m at 50 mcg./ml of DNA for thirty six hours, acquired several genetic characteristics similar to those of strain 15 m. The changes observed were:
   (1) streptomycin sensitivity to resistance.
   (2) absence to presence of streptomycin producing activity.

3. The induced new strain of strain G had resisted to 5,000 units of dehydrostreptomycin sulfate per ml. and produced 20 units of streptomycin in one ml of fermented broth.

4. The morphological specificities, physiological characters and phage sensitivity were not induced.

5. The DNA-preparations, acidic heat treated and DNA-ase treated, did not induce the genetic characters.

6. These results had suggested the possibility of the transformation. But, because there were other possibilities, for example, selection of mutants the present author could not draw any decisive conclusion for the hybridization mechanism.

Biochemical Studies on Storage Apples.

(p. *753~756*)

By Tatsuo OKAMOTO and Junko HARATA

(*Laboratory of Agricultural Products Technology, Faculty of Agriculture, Hirosaki University*)

As a fundamental study to increase the length of storage life of apples, a survey has been made of the metabolism of the apple during storage at 30° and 2°C.

With the deterioration of eating quality, remarkable changes have been shown especially in the rate of carbon dioxide production, the effect of adding 2,4-dinitrophenol on oxygen uptake determined on cut tissue by Warburg apparatus, decreases in acidity and hardness, and transformation of pectic snbstances.

Studies on the Carbohydrates of Tea: Part VI.

Hemicelluloses isolated from Tea-Wood. (p. *757~762*)

By Takashi MIZUNO, Genzo SAKATA and Tadao KINPYÔ

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University, Iwata*)

Studies were made concerning free sugars, pectic substances and hemicelluloses in the stem and the root of tea-wood.

The following results were obtained.

There was none of free sugar detected in the root, while in the stem, there were fructose (0.61%), glucose (4.80%) and saccharose (1.11%) for dry basis.

The yields of polysaccharides extracted with hot 0.5% ammonium oxalate solution, were 1.9% and 8.3% as regard the stem and the root respectively. The one extracted from the stem consisted of arabinose, galactose and galacturonic acid, and their molecular ratio was 1 : 7 : 2; this suggests that it is pectin. Another one extracted from the root constituted of galactose only: it is a kind of galactan.

Hemicellulose were extracted with cold (30°C) and hot (100°C) 5% sodium hydroxide solution. Their yields for dry basis were 3.5%, 1.0% (total 4.5%) from the stem, and 5.2%, 0.6% (total 5.8%) from the root, respectively. Most hemicelluloses were extracted in cold condition.

Component sugars of each hemicellulose were investigated by the qualitative and quantitative methods of paperchromatography. Xylose and glucose were identified as their components, and their molecular ratios were, on the average, xylose : glucose=11.2 : 1 in stem-hemicellulose, and 1 : 1.3 in root-hemicellulose.

Thus, it was confirmed that each hemicellulose isolated from the stem was a sort of glucoxylan, and that from the root was a sort of xyloglucan. Uronic acid was not detected in constructive sugar of both hemicelluloses. Physical and chemical properties of these hemicelluloses were investigated.

Polarographic Studies on Storage of Meats. Part I. Polarograms of Beef-Proteins (1). (p. *762~769*)

By Tetsujiro OBARA and Yasokichi OGASAWARA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Education*)

The polarographic wave given from respective protein may be particular to protein itself. Consequently, the polarography is a very excellent method to know the characteristic property of each protein. We have wished to use the polarographic method in studies on the storage of meats by γ-ray irradiation. Then, we have adopted the polarography in order to confirm the difference of polarographic behaviors given from proteins in raw meats more quickly and exactly than the methods used before.

In this experiments, we have examined the influence of electrolyzing conditions on polarographic waves of water and 7% NaCl extracts obtained from the raw beef, fresh and stored for three weeks at 15°C.

The experiments mentioned above result as follows:

(1) The maximum of the protein wave obtained from the raw beef is located at about -1.38 V for the first wave and about -1.55 V for the second wave.

(2) The wave-heights of beef-protein can be affected by the concentration of extract, $CoCl_2$ and NH_4Cl, but these curves of first and second waves have no crossing point in the range of our experiment.

(3) The crossing of the curves of the first and second wave affected by the NH_3-concentration and the temperature can be found in our experiments on the beef-protein.

(4) The waves of the water and 7% NaCl extracts which are obtained from the fresh beef differ very much from those of the stored beef.

Polarographic Studies on Storage of Meats. Part II. Polarograms of Beef-Proteins (2). (p. *770~778*)

By Tetsujiro OBARA and Yasokichi OGASAWARA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Education*)

It is well known that the change of the beef-constituent proceeds faster when the beef leaves alone in the room temperature. Consequently, it may be suggested that the polarographic wave of the protein from the beef in such condition varies in its shape with storage time, particularly if we use the beef by γ-ray irradiation. Then, in order to solve this suggestion we have examined the influence of the storage time on the polarographic wave of the beef-protein.

The experiments mentioned above result as follows:

(1) The maximum of the wave-height of the protein which is obtained from the beef stored at 15° and 25°C is located at about -1.38 V for the first wave and about -1.55 V for the second wave.

(2) In the case of either storage temperature of 15° and 25°C, there is the increase in the height of the first and the second wave from the water extract with the increase in storage time, contrarily the decrease in the wave height from 7% NaCl extract with storage time. The rate of such increrse and decrease in the wave height is especially rapid when the storage temperature is high.

(3) The polarographic wave of each protein from the following six parts of the beef—round, plate ribs, short loin, chuck rib poast, plate and tender loin—varies in its shape and height with the increase in storage time.

Studies on the Chromatography of Starches: Part VI: On the Amylose Content of Rice Starches during Maturity. (p. *778~781*)

By Motoji TAKI

(*Faculty of Agriculture, Mie University*)

Rice starches were prepared from the kernels at the various growth stages from the blooming to the harvest at weekly intervals. The amounts of amylose of these starches were determined by means of the descending paper chromatographic procedure by which the whole starches were separated into two fractions of amylose and amylopectin on the paper chromatograms developed with 40% $HClO_4$ solution in a chromatographic chamber containing iodine vapor, as described in the previous report (Part V).

The amylose content of the starches during maturity increased progressively until the dough stage of rice kernels, but after this stage remained almost unchanged towards the full maturity.

Studies on the Chromatography of Starches. Part VII. On the Efficacy of Amylose Precipitants for the Fractionation of Amylose and Amylopectin.

(p. *781~785*)

By Motoji TAKI

(*Faculty of Agriculture, Mie University*)

Amylose and amylopectin fractions were prepared from an ordinary rice starch by the similar method described by Schoch, using *n*-butyl alcohol, isoamyl alcohol, *n*-amyl alcohol, *n*-octyl alcohol, lauryl alcohol, thymol, cyclohexanol, methyl cyclohexanol and menthol an amylose precipitants. The absorption spectra of the solutions of these amylose fractions colored with iodine showed the disagreement in the wave lenghs at the maximum absorption and the optical densities. This fact was observed also on the amylopectin fractions. It was, therefore, considered that the amylose and amylopectin fractions were contaminated with amylopectin and amylose, respectively. Then, the purity of both fractions was investigated by the paper chromatographic procedure, as described in the previous report (Part V). The residual amylopectin in each amylose fraction was eluted with 40% $HClO_4$ solution from the paper chromatogram which had been developed with 40% $HClO_4$ solution by the descending technique in a chromatographic chamber containing iodine vapor, and its content was determined colorimetrically with iodine. The content of the residual amylose in each amylopectin fraction was determined by the same technique.

From the results of these experiments, it was confirmed that both amylose and amylopectin fractions were fractionated from the starch with *n*-amyl alcohol or thymol similar to the method described by Schoch in the higher purity than with other amylose precipitants.

Studies on Flavin. Part II. Mechanism of Protein Metabolism in Riboflavin Deficiency. Transaminase and Ribonuclease Activities of Rat Tissues. (1)

(p. *785～789*)

By Hisateru MITSUDA, Fumio KAWAI and Ben-ichiro TONOMURA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University*)

The flavin contents and some enzyme activities of riboflavin deficient rat tissues were compared with those of normal tissues. In kidney and heart, both of FMN and FAD had decreased to 60～70 per cent of those of normal after several weeks feeding without riboflavin, while in liver 85 per cent of FMN and 50 per cent of FAD were lost. At that time, hepatic glycolic acid oxidase and D-amino acid oxidase levels in the depleted group had dropped to 20～30 per cent of the normal, but about 50 per cent of hepatic xanthine oxidase remained.

The transaminase activities in kidney and heart of deficient rat were similar to or slightly higher than that of normal group, but in liver GOT activity was greatly higher and GPT activity a little than that of normal. It is interesting that the same value was found in the ratio of activities, GPT/GOT, in liver of riboflavin deficient rat and of rat fed on protein-free diet, although each activity behaved not identically in both cases.

The ribonuclease activities in whole homogenates of liver, kidney and muscle have shown no significant difference between normal and depleted animals.

Digestive Absorption of Protein by Swine. Part II. Effect of Antibiotic on Intestinal Absorption.

(p. *790～793*)

By Shikao ISHIKAWA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Mie University*)

It was the purpose of the present study to determine the extent of the protein absorption when chlortetracycline (as Aurofac 2A) was added to the ration, applying Cr_2O_3 indicator method, and to compare with that of non-chlortetracycline feeding in the previous paper.

(1) The antibiotic has no effect on the protein digestibility of the ration.

(2) It is regarded that the ingestion of the antibiotic has no effect on the pH values of the intestinal contents.

(3) By chlortetracycline feeding, the apparent absorption ratio lowers in the last section of small intestine and cecum, but does not change in the middle section of large intestine and rectum in comparison with the case of non-chlortetracycline feeding.

The Action of Amines on 1-Chloroacetyl-2,3,4,6-tetraacetyl-D-glucose.

(p. *794～797*)

By Yoshiyuki INOUE, Ikuo KARASAWA and Reiko NISHIO

(*Department of Biochemistry, College of Agriculture, Kyoto University*)

By the action of tri-, di- monochloracetic acid and phosphorus trichloride on β-pentaacetyl-D-glucose, we prepared three new substances, α-1-trichloroacetyl-2,3,4,6-tetraacetyl-D-glucose, m.p. 128° (uncorr.), $[\alpha]_D^{11}+98.1°$ (chlfm.), α-1-dichloroacetyl-2,3,4,6-tetraacetyl-D-glucose, m.p. 59～60° (uncorr.), $[\alpha]_D^{11}+95.9°$ (chlfm.), and α-1-monochloroacetyl-2,3,4,6-tetraacetyl-D-glucose, m.p. 125° (uncorr.), $[\alpha]_D^{21}+49.6°$ (chlfm.).

The α-1-trichloracetyl-2,3,4,6-tetraacetyl-D-glucose and α-1-dichloracetyl-2,3,4,6-tetraacetyl-D-glucose were converted into corresponding N-glucoside tetraacetates by condensation with amines in alcohol as polar solvent; on the contrary, corresponding tri-, dichloroacetic acid amides was obtained from these compounds by treatment with amines in benzene as nonpolar solvent, whereas α-1-monochloroacetyl-2,3,4,6-tetraacetyl-D-glucose was inert to amines in such of way.

Heterogeneity of α-Casein and Rennin Coagulation. Part III. (Studies on Milk Coagulating Enzymes. X)

(p. *797~800*)

By Kunio YAMAUCHI and Tomokichi TSUGO

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Influence of rennin on interaction between two fractions of Warner's α-casein, that is calcium insoluble fraction (α_R-casein) and calcium soluble fraction (crude κ-casein), was studied. It was shown that α_R-casein and para κ-casein (which was transformed from κ-casein by rennin specificially) formed complex irreversibly by warming the mixture to 35°. Evidences are as follows: (1) Disappearance of colloidal aggregates in para κ-fraction was observed after mixing it with α_R-fraction and warming to 35° by means of ultracentrifugal analysis (in phosphate buffer, pH 7.5, $\mu=0.2$). (2) Rapid disappearance of opalescence was confirmed by warming the mixture from 0° to 35° by measurement of transmittance (in 0.085 M NaCl solution, pH 6.6). (3) Two peaks in electrophoretic pattern of the mixture, which corresponded to α_R and para κ-caseins, were found to associate into one peak by warming previous to electrophoresis (in phosphate–NaCl buffer, pH 7.3, $\mu=0.1$). It was concluded that interaction between both fractions in unseparated α-casein was retained after rennin action.

Influence of Rennin on the Interaction between α- and β-Caseins; (Studies on Milk Coagulating Enzymes. XI)

(p. *801~806*)

Tomokichi TSUGO and Kunio YAMAUCHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Electrophoretic comparison of casein and paracasein was performed under various conditions in order to ascertain whether rennin affects on interaction between α- and β- caseins. The results are as follows. (1) Relative area of apparent β-casein peak both in ascending and descending patterns decreased with increasing protein concentration from about 0.3 to 3% (both in veronal and phosphate buffers of pH 7.3 and $\mu=0.1$). There is no difference between casein and paracasein in this tendency. Splitting in asceding α-peak of paracasein is more distinct in higher protein concentration or lower ionin strength. (2) Marked interaction is observed with rising electrophoresis temperature above about 20°. At 30° α- and β- peaks associate into one unhomogeneous peak. If pH is high (pH 11.1), however, α- and β-peaks are separated at such a hign temperature. In these characteristics no difference is found between casein and paracasein. (3) Abnormal and asymmetric pattern in calcium-containing buffer (veronal buffer containing 0.005 M calcium, pH 7.2, temperature 4°) will probably be ascribed also to interaction between α- and β- caseins. In this system the pattern of paracasein is considerable different from that of casein.

It is assumed that rennin does not split the α-, β casein complex into each component and that casein in milk is clotted as such a complex by rennin in usual condition.

Studies on the Chemical Combination between Lignin and Carbohydrate. Part VI. Dimethylformamide Extractive from Wheat Straw.

(p. *806~809*)

By Akira HAYASHI and Isamu TACHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University*)

Wheat straw meal was extracted with dioxane, 50% aq. acetic acid solution and DMF for several days. These extractives consisted of lignin and carbohydrate, and chemical combination was found between both constituents.

DMF extractive from wheat straw meal contained 8.6% methoxyl and the sugar constitution of its carbohydrate portion consisted of 17% galactose, 33% glucose, 28% arabinose and 21% xylose. Other two extractives had also almost the same sugar constitution as DMF extractive, although methoxyl (or lignin) content varried.

These sugar constitutions of lignin-carbohydrate compounds which were rich in galactose and arabinose differed from those of wellknown lignin-carbohydrate compounds such as xylo-lignin.

Studies on Amino-carbonyl Reaction of Milk Products. Part XXI. The Mechanisms of Degradation of Lactose. (3) On the Mechanisms of Transformation from Lactose to Monose in Heated Milk.

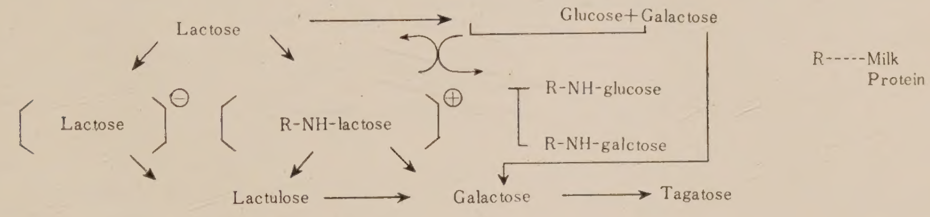
(p. *809~815*)

By Susumu ADACHI

(*Laboratory of Animal Products Technology, Faculty of Agriculture, Tohoku University, Sendai*)

The transformation of carbohydrates in milk heated at 110°C for 7, 13 and 23 hours has been studied by paper chromatography. From the results of the densitometric determination of the paper chromatograms, it has been established quantitatively that glucose is decreased at first and that galactose is remained in final stage of heating following the transformation of lactose to lactulose. It has been also determined by cystein-carbazol-sulfuric acid reaction that lactulose is formed rapidly to a certain level in response to the heating temperature in milk. From the hydrochloric acid hydrolysates of *p*-tolyllactosylamine and *p*-tolylisolactosylamine, lactulose, galactose, glucose, lactose (?), 5-hydroxymethylfurfural and *p*-toluidine have been detected. It has been shown that the transglycosylation can be occurred in *p*-tolyllactosylamine-hexose systems but cannot be occured in *p*-tolylglucosylamine-lactose system in alcoholic solution or water solution during heating. On the basis of these data, together with the conclusions of the previous papers, the scheme for the mechanisms of degradation of lactose has been proposed as follows.

It is appeared that Amadori rearrangement and Lobry de Bruyn-Alberda van Ekenstein transformation of carbohydrates are participated to the transformation of lactose in heated milk.



(continued from front cover)

M. NAKAMURA: Effects of Phosphatase and Phosphoglucomutase on the Determination of Phosphorylase Activities in Crude Extracts from Plant Materials. 383
M. NAKAMURA: The Sucrose-synthesizing Enzyme in the Beans. 398

Microbiology and Fermentation Industry

H. ŌNISHI: Studies on Osmophilic Yeasts. Part V. Factors Affecting Growth of Soy Yeasts and Others in the Environment of a High Concentration of Sodium Chloride. (II) 351
H. ŌNISHI: Studies on Osmophilic Yeasts. Part VI. Glycerol Production by the Salt-tolerant Yeasts in the Medium with High Concentrations of Sodium Chloride...359
S. NAKAMURA and T. SHIRO: Studies on Growth Inhibition of Hiochi-bacteria, Specific Saprophytes of Saké. Part IV. Hydroxyaspergillic Acid as a Growth Inhibitant against Hiochi-bacteria..418
K. SAKAGUCHI: Growth of *Pediococcus soyae* nov. sp. in Highly Concentrated Solutions of Inorganic Salts and Sugars. Studies on the Activities of Bacteria in Soy Sauce Brewing. Part VI. 438
K. SAKAGUCHI: P-Factor, a New Peptidic Growth Factor for *Pediococcus soyae*. Studies on the Activities of Bacteria in Soy Sauce Brewing. Part VII. 443

Note

H. ISHIKAWA and T. OKI: Biological Conversion of Shikimic Acid or Quinic Acid to Protocatechuic Acid, Gallic Acid and Aromatic Amino Acids. 451

Short Communications

T. KISAKI, M. IHIDA and E. WADA: A New Synthesis of N-Methylmyosmine. 454

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI A49
Errata 456

Printed by
KASAI PUBLISHING & PRINTING CO.
(*Pan-Pacific Press*)
Minato-ku, Tokyo, Japan
Tels. (50) 1421-24